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Chemotherapeutic Targeting of Fibulin-5 to Suppress Breast Cancer Invasion and Metastasis Stimulated by Transforming Growth Factor-beta

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14. ABSTRACT
The primary objective of this application is to establish how Fibulin-5 enhances the oncogenic activities of TGF-beta, particularly its ability to stimulate breast cancer invasion and metastasis. We hypothesized that inactivating Fibulin-5 function will prevent the conversion of TGF-beta from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-beta. Major findings of the past funding cycle include the ability of Fibulin-5 to (i) bind integrins on mammary epithelial cells (MECs) independent of its integrin-binding RGD motif; (ii) interact physically with TGF-beta and enhance its presentation to TGF-beta receptors; (iii) promote epithelial-mesenchymal transition in an integrin-independent manner in part by stimulating the expression of Cox-2, PAI-1, and MMP-9; (iv) enhance MEC proliferation by activating FAK and ERK1/2; and (v) induce MEC resistance to apoptosis and anoikis by stimulating NF-kappaB activation, by inducing survivin and xIAP expression, and by repressing TNF-alpha expression. Finally, we determined that Fibulin-5 expression is greatly augmented during breast cancer progression, particularly at the point when malignant MECs acquire metastatic phenotypes. This important finding implicates Fibulin-5 as a potential marker for breast cancer metastasis and reinforces the need to target Fibulin-5 chemotherapeutically in patients with metastatic disease.

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INTRODUCTION:

Breast cancer is the second leading cause of cancer death in women in the United States. Invasion and metastasis are the most lethal characteristics of breast cancer and the leading cause of breast cancer-related death. TGF-β normally inhibits breast cancer development by preventing mammary epithelial cell (MEC) proliferation, or by inducing MEC apoptosis. Mammary tumorigenesis counteracts the tumor suppressing activities of TGF-β, thus enabling TGF-β to stimulate breast cancer invasion and metastasis. Fundamental gaps exist in our knowledge of how malignant MECs overcome the cytostatic actions of TGF-β, and of how TGF-β stimulates the development and progression of mammary tumors. These knowledge gaps have prevented science and medicine from implementing treatments effective in antagonizing the oncogenic activities of TGF-β in developing and progressing breast cancers. We recently discovered that the expression and activity of the TGF-β gene target, Fibulin-5 (FBLN5), potentiates TGF-β stimulation of invasion and epithelial-mesenchymal transition (EMT) in normal and malignant MECs *in vitro*, and more importantly, enhances the growth and pulmonary metastasis of mammary tumors in mice. Interestingly, we find that FBLN5 incorporates into active TGF-β receptor complexes in a β3 integrin-dependent manner, an event associated with the activation of intracellular signaling by TGF-β. Based on these and other compelling findings, we hypothesized that inactivating FBLN5 function will prevent the conversion of TGF-β from a suppressor to a promoter of breast cancer growth and invasion, thereby alleviating breast cancer development and progression stimulated by TGF-β. The goals of this project are to determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes, and to determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs. Finally, we will determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression *in vivo*. These studies are important because they will *(i)* provide valuable information on how breast cancers develop and progress, and on how TGF-β promotes these processes; *(ii)* identify the signaling mechanisms and systems that mediate the oncogenic nature of TGF-β; and *(iii)* identify FBLN5 antagonists capable of alleviating the oncogenic activities of TGF-β, as well as establish their effectiveness in preventing breast cancer progression stimulated by TGF-β. Moreover, application of our findings will enable science and medicine to one day improve the prognosis and treatment of patients with metastatic breast cancer

BODY:

Overview and General Findings: The specific aims of the proposed research have not been modified. Indeed, our recently published manuscript in the journal *Carcinogenesis* [1] clearly established the importance of FBLN5 in promoting epithelial-mesenchymal transition (EMT) in normal and malignant mammary epithelial cells (MECS). Equally important, we showed that FBLN5 expression greatly enhanced the ability of TGF-β to stimulate EMT, as well as promoted its oncogenic activities in normal and malignant MECs both *in vitro* and *in vivo*. Clearly, elucidating the molecular mechanisms that enable FBLN5 to enhance oncogenic TGF-β signaling has tremendous potential to neutralize the metastasis promoting activities of this multifunctional cytokine, and as such, to ultimately improve the clinical course of breast cancer patients with metastatic disease.

Data in the scientific literature has recently established the essential role of TGF-β in regulating the activities of breast cancer-associated fibroblasts and stromal components [2-5]. Indeed, mounting evidence indicates that TGF-β promotes breast cancer progression in part *via* its reprogramming of MEC microenvironments and their cellular architectures. Moreover, TGF-β also induces desmoplastic and fibrotic reactions that elicit the formation of tense, rigid tumor microenvironments that *(i)* enhance the selection and expansion of developing mammary neoplasms, particularly that of late-stage metastatic cells, and *(ii)* predict for poor clinical outcomes in breast cancer patients. Our previous published studies established FBLN5 as an important stromal-produced secreted factor that regulates tumor development in mice [6-9]. Thus, we characterized changes in the fibroblast transcriptome elicited by FBLN5, or by FBLN5 plus TGF-β. Microarray analyses identified 1181 genes whose expression is regulated by FBLN5, and an additional 1675 genes whose expression is regulated by

TGF-β. Differential expression of 14 individual genes was verified by semi-quantitative real-time PCR. Downregulated FBLN5 gene targets included *a)* BB503935; *b)* pleckstrin-homology domain-containing family A member; *c)* transglutaminase-2; and *d)* Rho GTPase activating protein 24. Upregulated FBLN5 gene targets included *a)* BB533736; *b)* BB831146; *c)* HoxD9; *d)* thrombospondin-1; *e)* collagen type XI; *f)* angiopoietin-1; *g)* cysteine-rich protein 61; *h)* Dkk3; *i)* fibromodulin; and *j)* HoxD10. Oncomine analyses showed the expression of fibromodulin to be upregulated in human breast cancers, and as such, we further characterized the activities of this novel FBLN5 gene target. In doing so, we found that fibromodulin expression greatly enhanced the coupling of TGF-β to Smad2/3 and AP-1 activation, while simultaneously abrogating both basal and TGF-β-stimulated NF-κB activation in fibroblasts. Importantly, we observed fibromodulin expression to stabilize that of the NF-κB inhibitory protein, IκBα. We further determined that fibromodulin stabilized IκBα expression by activating JNK and CK-II, which inactivate calpain and its proteolytic activity against IκBα. Thus, in addition to inhibiting NF-κB activity in fibroblasts, the activation of this fibromodulin-dependent pathway promotes apoptosis in fibroblasts. Even more strikingly, Oncomine analyses showed the expression of fibromodulin to be reduced at metastatic sites relative to nonmetastatic lesions in gastric cancers, head and neck cancers, and sarcomas. These analyses also found aberrantly low fibromoduliun expression to associate with reduced overall survival rates in patients with cancers of the brain, breast, lung, and blood. Our findings related to this novel FBLN5:fibromodulin signaling axis have been accepted by the *Journal of Biological Chemistry* pending successful completion of minor visions. *A copy of the unedited and accepted version of this manuscript is provided in the Appendix materials*. **Clinically, chemotherapeutic targeting of this pathway may offer novel inroads into alleviating the oncogenic activities of TGF-β in breast cancer stroma.**

Based on our findings presented below, we remain convinced that our analyses of noncanonical and oncogenic effectors targeted by FBLN5 and TGF-β will enable the development of safer, more directed chemotherapies capable of phenotypically normalizing and reverting the malignant behaviors of developing and progressing breast cancers.

Task-Specific Findings:

Task 1: ***Determine the molecular mechanisms that mediate incorporation of FBLN5 into active TGF-β receptor complexes.*** We previously engineered normal NMuMG and metastatic 4T1 cells to stably express β3 integrin or its inactive mutant, D119A-β3 integrin [10-12]. Our previously published studies demonstrated the function of β3 integrin in promoting oncogenic TGF-β signaling, including its ability to stimulate EMT and pulmonary metastasis of breast cancer cells [10-12]. We recently introduced wild-type FBLN5 and its RGE-mutant, which we demonstrated previously to prevent FBLN5 from ligating integrins on endothelial cells [7]. The functional characteristics of these FBLN5 and β3 integrin manipulations on MEC behavior in response to TGF-β are quite interesting and will be discussed below (*see Task 2).* With respect to the primary objective of Task 1 – *i.e.,* to identify the molecular determinants that mediate incorporation of FBLN5 into active TGF-β receptor complexes, and more importantly, to determine the impact of disrupting the formation of these complexes on normal and malignant MEC response to TGF-β – our preliminary data indicate that FBLN5 is capable of binding β3 integrin on MECs independent of its integrin-binding RGD motif. Indeed, we find that MEFs derived from FBLN5-deficient embryos respond poorly to TGF-β, and that re-expression of either wild-type FBLN5 or RGE-FBLN5 molecules in these FBLN5-deficient MEFs significantly enhance MEF response to TGF-β. Thus, our findings to date suggest that FBLN5 may incorporate into TGF-β receptor complexes independent of traditional integrin-binding activities. Alternatively, FBLN5 may incorporate into TGF-β receptor complexes in a manner wholly independent of β3 integrin. With respect to the former possibility, we now are optimizing the expression and purification systems necessary to isolate various recombinant FBLN5 mutants, including full-length wild-type and RGE-mutant FBLN5 molecules, as well as those mutants that lack the N-terminal Pro-rich domain (*i.e.,* ΔPro), the entire N-terminal domain (*i.e.,* ΔNT), and the entire globular C-terminal domain (*i.e.,* ΔCT) or those that only contain the N-terminal (*i.e.,* NT-FBLN5) or C-terminal (*i.e.,* CT-FBLN5). FBLN5 mutants found to incorporate into TGF-β receptor complexes will then be subjected to gross- and fine-

deletion analyses, followed by Ala-scanning mutagenesis to elucidate the molecular determinants that mediate FBLN5 association with TGF-β receptors. We fully expect to possess engineered FBLN5 molecules that are incapable of supporting oncogenic TGF-β signaling by the completion of Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3.

Because our initial studies of FBLN5 incorporation into TGF-β receptor complexes showed that wild-type and RGE-FBLN5 were both capable of capturing β3 integrin in immunocomplex assays, we began to consider the possibility that FBLN5 may incorporate into TGF-β receptors in an integrin-independent fashion. In support of this notion, we found that FBLN5 bears striking homology to members of LTBP (latent TGF-β-binding proteins) family of proteins, particularly in their calcium-binding EGF-like repeats. Thus, we hypothesized that FBLN5 may bind directly to TGF-β, which then pulls FBLN5 into TGF-β receptor complexes. Accordingly and quite surprisingly, we used three separate and distinct binding protocols to show unambiguously that FBLN5 does indeed interact physically with active TGF-β independent of whether FBLN5 can bind to integrins (*i.e.,* wild-type FBLN5 and RGE-FBLN5 bind indistinguishably to active TGF-β). **This finding represents a major advance for TGF-β and FBLN5 biologists, and may in fact explain why FBLN5-deficient MEFs are unresponsive to TGF-β**. Indeed, our findings indicate that FBLN5 may function in binding directly to TGF-β and facilitating its presentation and/or incorporation to inactive TGF-β receptor complexes, resulting in enhanced transmembrane signaling initiated by TGF-β. Accordingly, MECs engineered to overexpress FBLN5 exhibit significantly elevated levels of Smad2/3 activity as compared to their GFP-expressing counterparts, a finding consistent with FBLN5 functioning to present and enhance autocrine TGF-β signaling in normal and malignant MECs. We have now engineered MECs to produce various FBLN5 mutants to map the domains operant in mediate its interaction with TGF-β. After affirming which regions of FBLN5 bind TGF-β1, we will immediately generate FBLN5 mutants that lack this domain/motif to assess how preventing FBLN5 from binding TGF-β impacts normal and malignant MEC response to TGF-β both *in vitro* and *in vivo.* As above, we fully expect to complete this exciting and important task during Year 2, and to complete a thorough characterization of their impact on TGF-β signaling and breast cancer cell behavior during Year 3

Task 2: ***Determine the role of FBLN5 in mediating β3 integrin and Src activation, leading to oncogenic signaling by TGF-β in normal and malignant MECs.*** The primary objective of Task 2 is to identify FBLN5 effectors operant in mediating oncogenic signaling by TGF-β. In this regard, we have found that wild-type and RGE-FBLN5 are both capable of promoting partial EMT phenotype in normal MECs (**Fig. 1**). Moreover, we find FBLN5 expression to be significantly upregulated in 4T1 progression series, which is an established mouse model of triple-negative breast cancer. Interestingly, we also observed the combination of FBLN5 and β3 integrin to significantly enhance the proliferative potential of normal MECs, a response that was not recapitulated in MECs co-expressing RGE-FBLN5 and β3 integrin. In addition, the combined expression of FBLN5 and β3 integrin greatly attenuated the sensitivity of MECs to the cytostatic activities of TGF-β. The enhanced response of MECs to FBLN5 also correlated with its ability to significantly augment the activation of FAK and ERK1/2 in these same cells. Thus, FBLN5 expression induced by TGF-β in normal and malignant MECs appears to play a significant role in mediating its growth promoting activities in MECs. We recently developed the only *in vitro* assay that wholly recapitulates the phenomena underlying the "TGF-β Paradox" during mammary tumorigenesis [13, 14], which converts the actions of TGF-β from that of a tumor suppressor to a tumor promoter (*see* [15-18]). We exploited this unique 3D-organotypic culture system to further explore the function of FBLN5 in promoting an invasive phenotype in normal MECs. In doing so, we propagated parental NMuMG cells or their derivatives engineered to stably express either FBLN5, β3 integrin, or both transgenes in combination in the absence or presence of TGF-β under compliant or rigid 3D-organotypic culture conditions. **Figure 2** shows that relative to parental (*i.e.,* GFP/YFP) NMuMG cells, those engineered to express β3 integrin formed substantially larger (by 275%) and densely packed acinar structures, while those expressing FBLN5 formed substantially smaller (by 65%) organoids. Interestingly, NMuMG cells that expressed both transgenes formed acinar

structures whose size was also substantially larger (by 195%) than their parental counterparts and in many respects resembled those of β3 integrin-expressing NMuMG cells (**Fig. 2**). Thus, β3 integrin stimulates acinar growth, while FBLN5 inhibits this event in a manner that can be neutralized by expression of β3 integrin. Despite the dramatic differences in their relative growth rates, all four NMuMG derivatives readily responded to the cytostatic activities of TGF-β and formed diminutive organoids when propagated in continued presence of this cytokine (**Fig. 2**). When cultured under rigid conditions, all NMuMG derivatives acquired a branched morphology that was potentiated by β3 integrin expression (**Fig. 2**). Quite surprisingly, FBLN-expressing NMuMG cells formed unique linear and irregularly-branched structures indicative of a highly invasive phenotype (**Fig. 2**). Moreover and in contrast to their parental and β3 integrin-expressing expressing counterparts, FBLN5-expressing NMuMG cells were uniquely resistant to the apoptotic inducing activities of TGF-β under rigid microenvironmental conditions (**Fig. 2**). Moreover, the survival promoting activities of FBLN5 are wholly coupled to the ability of FBLN5 to activate Src *(data not shown*). Taken together, these new findings offer some potentially important insights into the role of FBLN5 in MECs, suggesting that FBLN5 may function as an inhibitor of acinar growth and development in compliant microenvironments. However, these findings also suggest that FBLN5 may sense and respond to mechanically rigid and tense microenvironmental conditions, resulting in the acquisition of invasive and survival phenotypes.

Along these lines and in stark contrast to its effects in fibroblasts [9], we find that FBLN5 greatly enhances basal and TGF-β-stimulated NF-κB activity in normal (**Fig. 3**) and malignant *(data not shown)* MECs in part *via* promoting increased degradation of IκBα (**Fig. 3**). Moreover, these FBLN5-dependent activities require signaling inputs initiated by Src *(data not shown)*. Indeed, we recently found that EMT induced by TGF-β initiates a pro-survival gene expression profile *(data not shown)*, such that MECs that survive the EMT process are more resistant to apoptosis and anoikis. Given our published work that FBLN5 promotes EMT in normal and malignant MECs [1], we reasoned that FBLN5 expression would also promote survival signaling in these same cells. Accordingly, we now find that FBLN5 greatly suppresses TNF-α expression (by 90%) in normal MECs *(data not shown)*, while simultaneously stimulating that of the *(i)* survival factors, survivin and xIAP *(data not shown)*; *(ii)* angiogenic and EMT molecule, Cox-2 *(data not shown)*; *(iii)* pro-metastatic molecule, PAI-1 *(data not shown)*; and *(iv)* pro-invasion and EMT-molecule, MMPs 2, 3, and 9 *(see below)*. In addition, we further observed FBLN5 expression to be sufficient in inhibiting Caspase-3/7 activation by TNF-α in normal MECs (**Fig. 4**). In addition, we have begun manipulating the expression of these FBLN5 gene targets in normal and malignant MECs to access their role in regulating MEC response to TGF-β both *in vitro* and *in vivo.* Initial targets are members of the MMP family of proteases. Indeed, we observed FBLN5 to strongly induce the expression of MMPs 2, 3, and 9 in normal (**Fig. 5**) and metastatic (**Fig. 6**). Interestingly, whereas the ability of FBLN5 to activate NF-κB transpires through an integrin-independent pathway (**Fig. 3**), the coupling of FBLN5 to MMP expression clearly requires signaling inputs from integrins. For instance, **Figure 7** shows that FBLN5, but not its mutant RGE counterpart (*i.e.,* cannot ligate integrins), readily and potently induces the expression of MMPs 2, 3, and 9. Surprisingly, expression of β3 integrin inhibited the ability of FBLN5 to promote MMP expression, a reaction that was not recapitulated by expression of the nonfunctional β3 integrin mutant, D119A-β3 integrin. Thus, while FBLN5 clearly binds β3 integrin, this event serves to neutralize MMP expression stimulated by FBLN5. Mechanistically, the ability of FBLN5 to induce MMP expression in normal MECs transpires through the activation of MAP kinases and Src, as well as require Ca^{++}-dependent signaling inputs (**Fig. 8**). Our findings in **Figure 9** wholly support this idea and also show that the integrin effectors, FAK and Pyk2, are necessary for FBLN5 stimulation of MMP expression in NMuMG cells. In addition to binding to αvβ3 integrin, FBLN5 also ligates αvβ5 and α9β1 integrins, suggesting that the coupling of FBLN5 to MMP expression way proceed through other integrin heterodimers operant in binding FBLN5. Along these lines, we have recently determined that FBLN5 induces MMP expression through a β1 integrin- and ERK1/2-dependent pathway (**Fig. 10**). Indeed, neutralizing antibodies against β3 integrin were ineffective in altering the coupling of FBLN5 MMP expression, which contrasts sharply to the dramatic reduction in MMP expression observed following administration of either neutralizing β1 integrin antibodies (**Fig. 10**), or following cellular depletion of β1 integrin expression (**Fig. 11**).

These findings are a major advance to the fibulin field, and we now are rapidly extending these findings to the aforementioned normal and malignant MECs engineered to express all combinations of wild-type and mutant FBLN5 and β3 integrin molecules.

Task 3: ***Determine whether interdicting FBLN5 function abrogates the oncogenic activities of TGF-β and prevents its stimulation of breast cancer progression in vivo***. The primary objective of Task 3 is to establish the effectiveness of abolishing FBLN5 function and its subsequent incorporation into active TGF-β receptor complexes to prevent breast cancer progression and metastasis induced by TGF-β. As mentioned above, this past year saw us identify a variety of novel FBLN5 gene targets, as well as uncover two potentially important tumor promoting functions for FBLN5, namely its ability to facilitate the presentation of TGF-β to its receptors and its potential to induce survival signaling in normal and malignant MECs. In initial pilot studies, we determined whether overexpression of FBLN5 in NMuMG cells would be sufficient to induce their formation of mammary tumors in nude mice. Unfortunately, tumor development was not induced by FBLN5 expression, indicating that aberrant expression of the ECM molecule is not sufficient to transform MECs and drive tumor development. However, these findings are consistent with a role of FBLN5 in driving EMT (**Fig. 1**; [1]), invasion (**Fig. 9**; [1]), and metastasis in late-stage breast cancers [1]. Accordingly, FBLN5 expression is increased robustly in the murine 4T1 progression series from weakly tumorigenic 67NR cells to fully metastatic 4T1 cells (**Fig. 1**). In the next year, we will rapidly test these FBLN5 functions using malignant, nonmetastatic 67NR and malignant, highly metastatic 4T1 cells that will be engineered to stably express FBLN5 mutants that fail to bind and present TGF-β to its receptors, as well as those construct derivatives of these breast cancer cell lines whose expression of FBLN5 target genes has been positively and negatively manipulated. Afterward, the impact of these manipulations on primary tumor growth and metastasis will be assessed in syngeneic Balb/C mice.

KEY RESEARCH ACCOMPLISHMENTS:

- Mammary tumorigenesis upregulates FBLN5 expression, particularly at the point when breast cancer cell acquire metastatic phenotypes
- A novel FBLN5 gene signature has now been identified and established
- The FBLN5 gene target, fibromodulin, suppresses NF-κB activity by stabilizing IκBα expression
- Stabilization of IκBα transpires via JNK and CK-II activation, which conspire to inactivate calpain and its proteolytic activity against IκBα
- Activation of this fibromodulin signaling axis promotes apoptosis
- FBLN5 interacts with β3 integrin in an RGD-independent fashion
- FBLN5 binds TGF-β, leading to its enhanced presentation to TGF-β receptors and elevated autocrine TGF-β signaling in normal and malignant MECs
- FBLN5 and β3 integrin promote normal and malignant MEC proliferation, a cellular response coupled to FAK and ERK1/2 activation by FBLN5
- RGE-FBLN5 and β3 integrin fail to induce MEC proliferation
- FBLN5 induces survival signaling in normal and malignant MECs in part by strongly activating NF-κB
- Survival signaling by FBLN5 is also coupled to its ability to suppress TNF-α expression, and to induce that of survivin and xIAP
- FBLN5 potentially induces breast cancer cell EMT, migration, and invasion by upregulating the expression of Cox-2, PAI-1, and MMPs 2, 3 and 9
- β1 integrin is essential for coupling FBLN5 to ERK1/2 activation, which subsequently induces MMP expression in normal and malignant MECs.

REPORTABLE OUTCOMES:

Schiemann Laboratory Publications Acknowledging Support of BC084651:

Keshamouni, V.G. and **Schiemann, W.P.** (2009) EMT in Tumor Metastasis: A Method to the Madness. **Future Oncology** *5,* 1109-1111.

Wendt, M.K., Allington, T.M. and **Schiemann, W.P.** (2009) Mechanisms of epithelial-mesenchymal transition by TGF-β in normal and malignant cells. **Future Oncology** *5,* 1145-1168.

Tian, M. and **Schiemann, W.P.** (2009) The TGF-β paradox in human cancer: An update. **Future Oncology** *5,* 259-271.

Wendt, M.K., Smith, J.A. and **Schiemann, W.P.** (2009) p130Cas is required for mammary tumor growth and TGF-β-mediated metastasis through regulation of Smad2/3 activity. **Journal of Biological Chemistry** *284,* 34145-34156 *See Faculty of 1000 (http://f1000biology.com/article/id/1168476)

Taylor, M.A., Parvani, J.G. and **Schiemann, W.P.** (2010) The pathophysiology of EMT stimulated by TGF-β. **J Mammary Gland Biol Neoplasia** *15:* 169-190.

Wendt, M.K., Smith, J.A. and **Schiemann, W.P.** (2010) TGF-β-induced epithelial-mesenchymal transition facilitates oncogenic epidermal growth factor receptor signaling in breast cancer. **Oncogene** *29:* 6485-6498.

Tian, M., Neil, J.R. and **Schiemann, W.P.** (2010) TGF-β and the hallmarks of cancer. **Cell Signal** *22: in press.* PMID: 20940046

Allington, T.M. and **Schiemann, W.P.** (2011) The Cain and Abl of epithelial-mesenchymal transition and TGF-β in mammary epithelial cells. **Cells Tissues Organs** *193:* 98-113.

Lee, Y-H. and **Schiemann, W.P.** (2010) Fibromodulin suppresses nuclear factor-κB activity by inducing the delayed degradation of IκBα *via* a JNK-dependent pathway coupled to fibroblast apoptosis. **J Biol Chem** *286,* in press. PMID: 21156791.

Invited Seminars Presented by Dr. Schiemann Acknowledging Support of BC084651:

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **UC-Davis Cancer Center,** Sacramento, CA. (May 14, 2009).

Schiemann, W.P. (2009) Oncogenic TGF-β signaling in breast cancer. **Case Comprehensive Cancer Center,** Cleveland, OH. (July 16, 2009).

Schiemann, W.P. (2009) Activated Abl kinase inhibits oncogenic TGF-β signaling, EMT, and tumorigenesis in mammary tumors. **The EMT International Association's 4th International Meeting on "Epithelial-Mesenchymal Transition,"** Tucson, AZ. (September 23, 2009).

Schiemann, W.P. (2009) The Abl and Cain of TGF-β signaling. **Department of Pharmacology,** Case Western Reserve University, Cleveland, OH. (October 5, 2009).

Schiemann, W.P. (2010) The Cain and Abl of EMT and TGF-β signaling in mammary epithelial cells. *AACR Special Conference on "EMT and Cancer Progression and Treatment,"* Arlington, VA. (March 1, 2010).

Schiemann, W.P. (2010) Oncogenic TGF-β signaling in breast cancer. *University of Tennessee, Comparative and Experimental Medicine Research Seminar Series at the UT College of Veterinary Medicine*, Knoxville, TN (October 11, 2010).

Schiemann, W.P. (2010) Oncogenic TGF-β signaling in breast cancer. *Eppley Cancer Institute, University of Nebraska Medical Center,* Omaha, NE (October 28, 2010).

Schiemann, W.P. (2010) The Cain and Abl of EMT and TGF-β signaling in Breast Cancer. *Translational Genomics Research Institute (TGen),* Phoenix, AZ (November 8, 2010).

Schiemann, W.P. (2010) Oncogenic TGF-β Signaling in breast cancer. *Lerner Research Institute, Cleveland Clinic Foundation,* Cleveland, OH (December 7, 2010).

CONCLUSION:

Our findings have clearly established new biological and pathological paradigms for FBLN5 and TGF-β. Importantly, we continue to *(i)* elucidate the mechanisms whereby FBLN5 induces oncogenic

TGF-β signaling in normal and malignant MECs, and *(ii)* identify the FBLN5 effectors that contribute to the invasive and metastatic properties of TGF-β. Equally importantly, our findings have provided the first FBLN5 gene signature that underlies its biological activities, and this dataset has already uncovered fibromodulin as a novel FBLN5 gene target that regulates fibroblast survival. Our findings that FBLN5-deficient MEFs are largely unresponsive to TGF-β is exciting and may in fact be explained by our demonstration that FBLN5 binds directly to TGF-β, leading to its presentation to TGF-β receptors and the enhanced activation of autocrine TGF-β signaling in normal and malignant MECs. Our findings have also identified several novel FBLN5 effectors whose activity contributes to oncogenic TGF-β signaling. Given our recent finding that developing and progressing mammary tumors significantly upregulate their expression of FBLN5 at the point at which these tumors become metastatic, our results clearly establish FBLN5 as a new and potentially important biomarker to detect and track metastatic disease in patients with breast cancer. Moreover, the ability of FBLN5-deficiency to significantly attenuate cellular responses to TGF-β suggest that measures capable of antagonizing FBLN5 function may alleviate the initiation of oncogenic TGF-β signaling. Indeed, successful identification and implementation of FBLN5 molecules that are unable to bind and present TGF-β to its receptors on metastatic breast cancer cells holds tremendous potential to alleviate metastatic disease in breast cancer patients. Thus, translation of our findings will provide a novel set of biomarkers comprised of FBLN5 and its effectors that will be capable of predicting whether or not malignant MECs possess metastatic phenotypes. In addition, our findings will offer new inroads to target these metastatic lesions via employment of FBLN5 mutants that will suppress oncogenic TGF-β signaling in breast cancer cells. Collectively, we envision that further developing these reagents and clinical protocols will play a significant role in developing a "personalized medicine" approach tailored to treat individuals with metastatic breast cancer.

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APPENDICES:

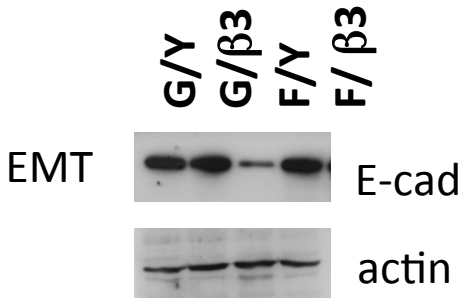
Contains Schiemann Laboratory Publications Acknowledging Support of BC084651 as Listed Under "REPORTABLE OUTCOMES"

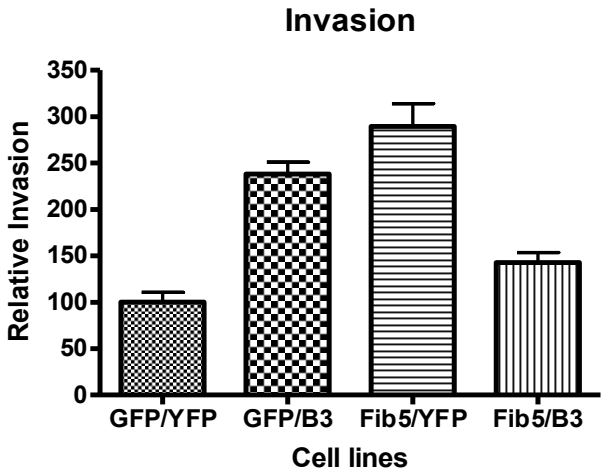
- Supporting Figures 1-11
- Accepted and Unedited manuscript by Yong-Hun Lee and William P. Schiemann to *Journal of Biological Chemistry*
- Published Schiemann Lab papers acknowledging DoD BC084651 support.

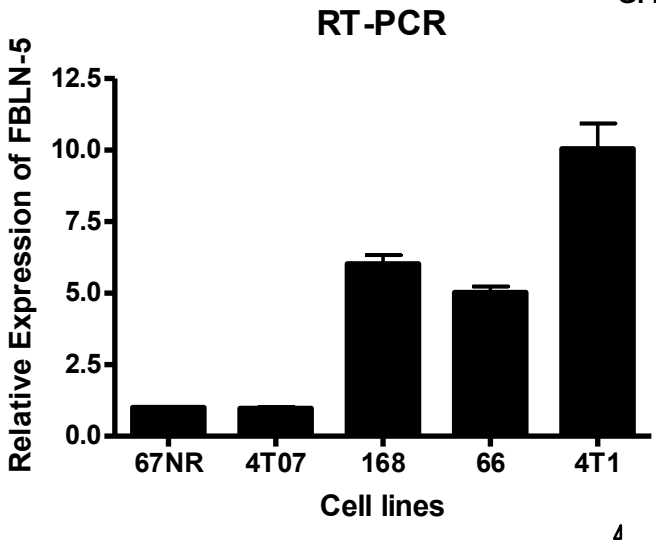
Figure 1

FBLN5-expressing MECs Exhibit EMT and Invasive Phenotypes:

Role of FBLN5 in Breast Cancer Progression?



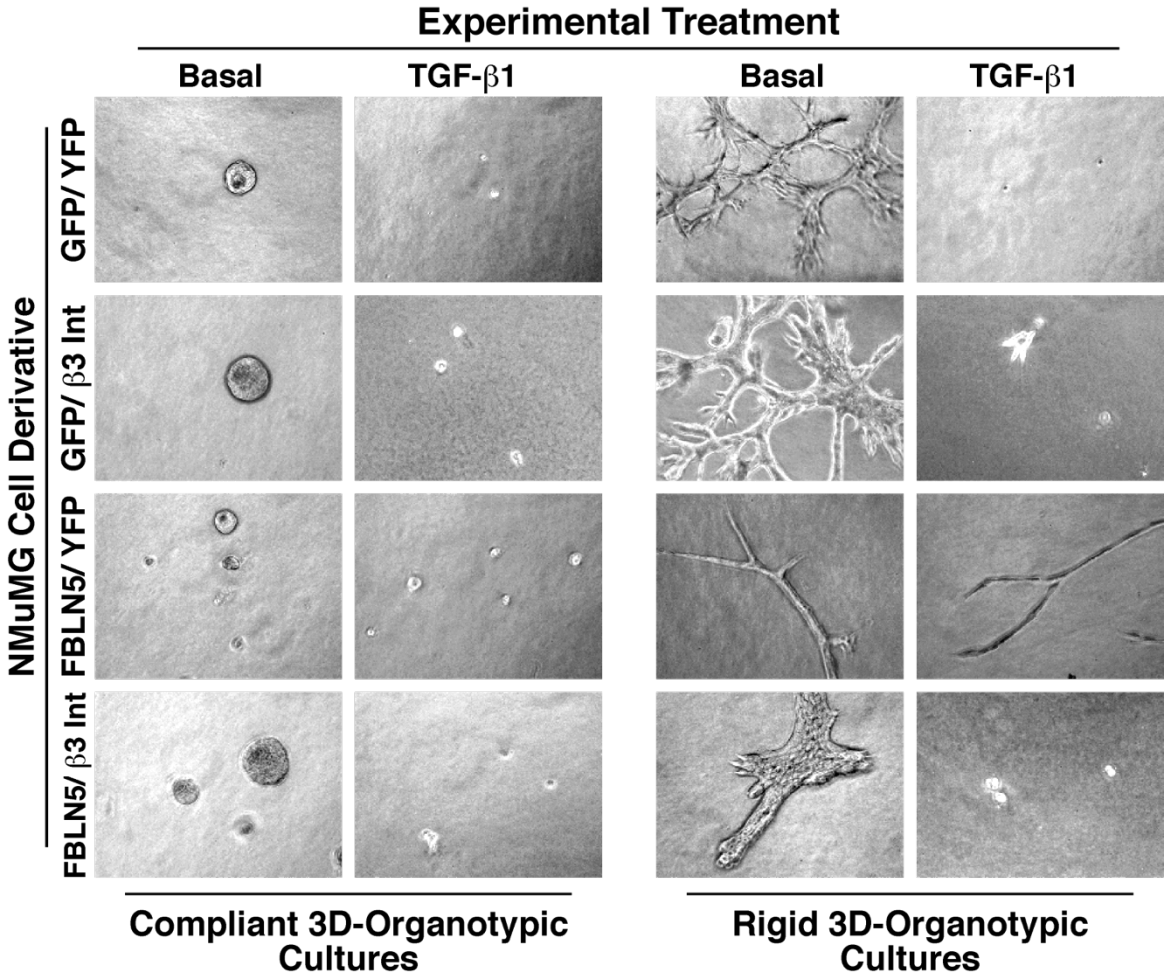




Tumorigenicity

Figure 2

FBLN5 Induces Invasive Morphologies and Survival of MECs in Rigid Environments



NMuMG derivatives were cultured in the absence or presence of TGF-β1 (5 ng/ml) for 8 days in 3D-organotypic Cultrex matrices supplemented without (*i.e.,* compliant) or with type I collagen (3 mg/ml; *i.e.,* rigid). Bright-field images were captured and representative images from 3 independent experiments are shown.

Figure 3

FBLN5 Stimulates NF-κB Activity Independent of Integrin Binding in NMuMG Cells: *Targeting IκBα Destruction*

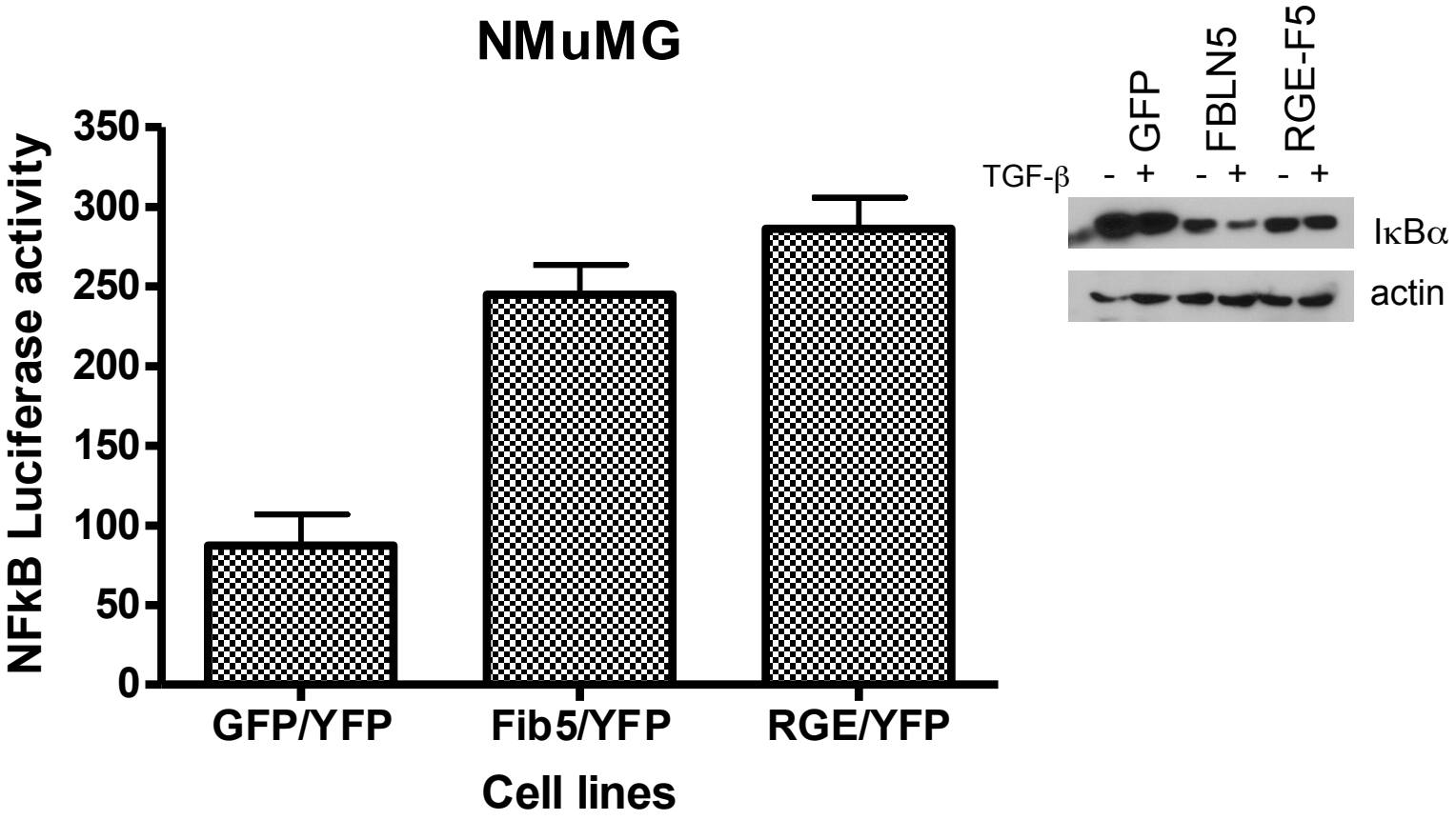


Figure 4

FBLN5 Suppresses the Coupling of TNF-α to Caspases 3 & 7 in NMuMG Cells

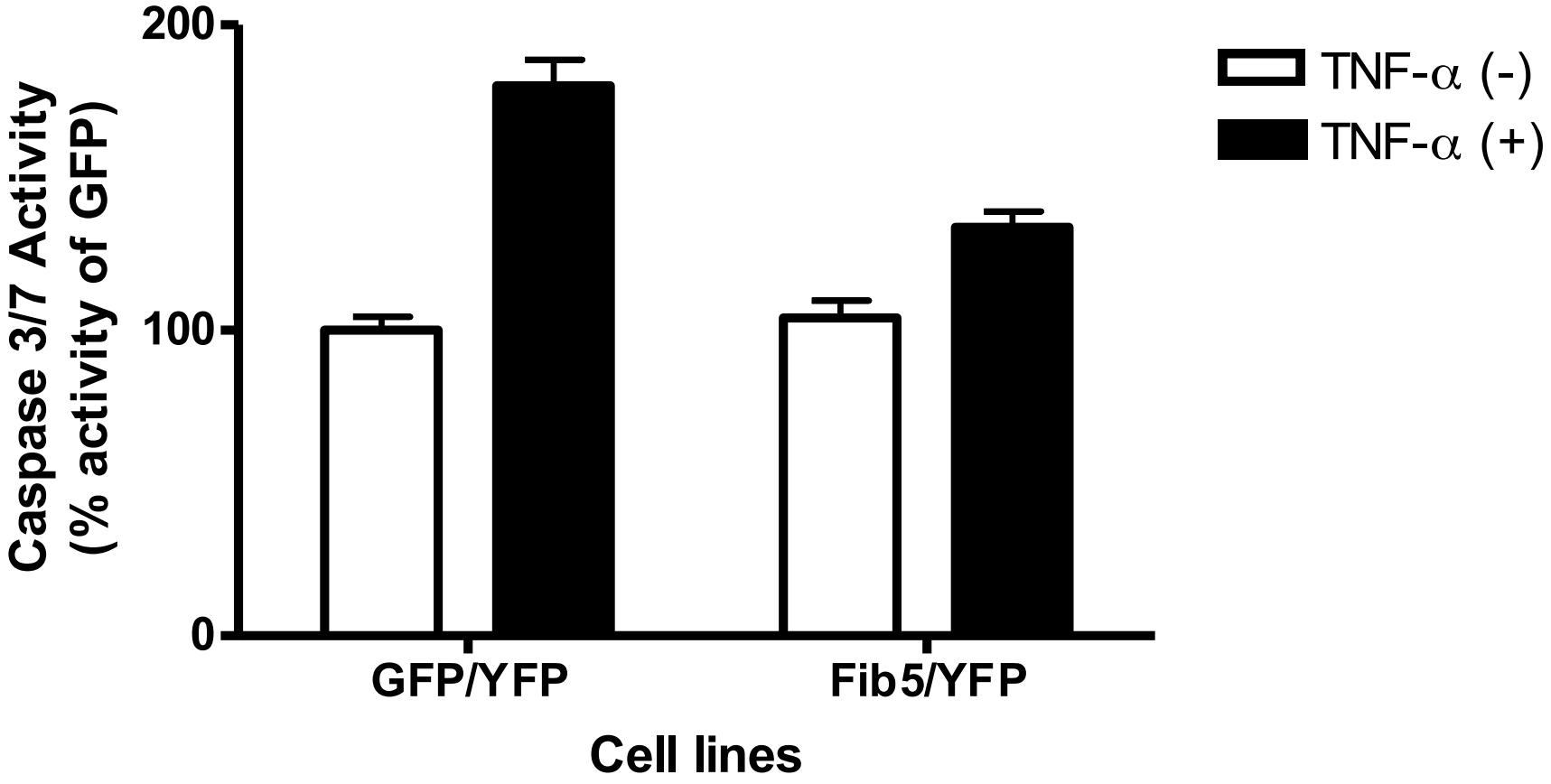


Figure 5

FBLN5 is a Potent Inducer of MMP Expression in Normal NMuMG Cells

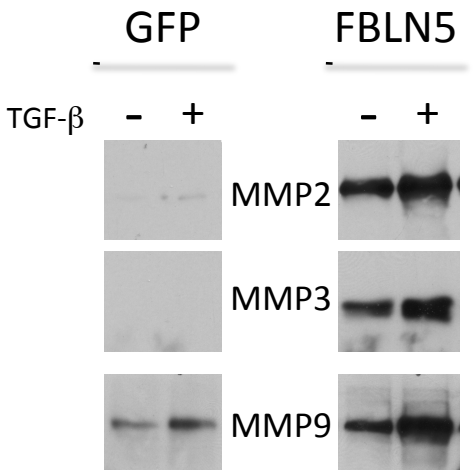


Figure 6

FBLN5 is a Potent Inducer of MMP Expression in Metastatic MECs

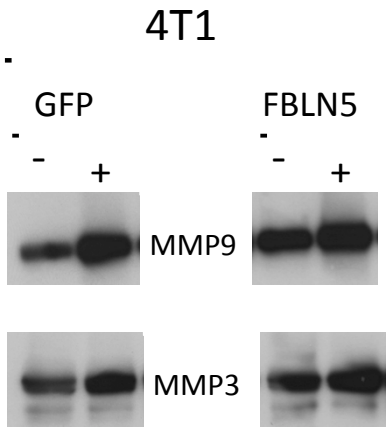
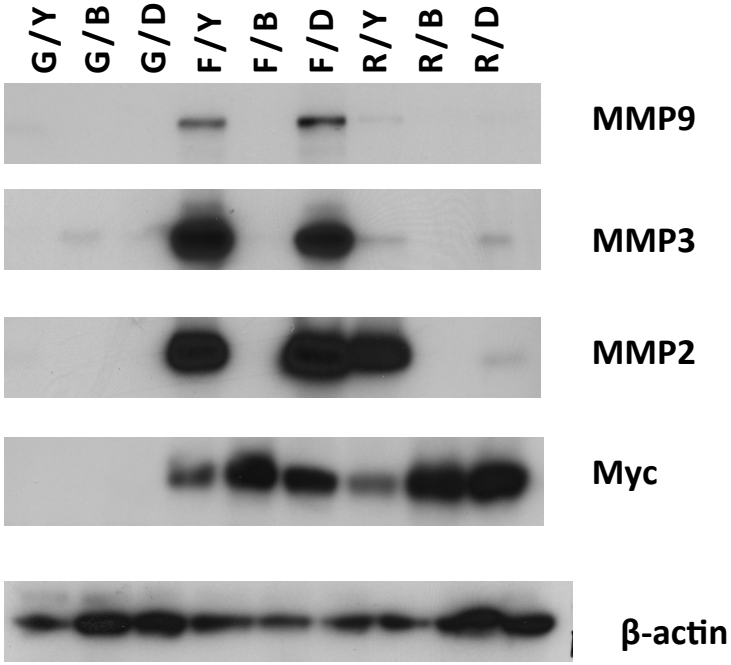


Figure 7

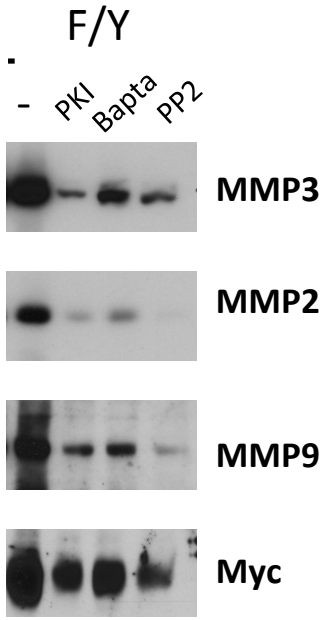
β3 integrin masks FBLN5 stimulation of MMP Expression in NMuMG cells



G = GFP
Y = YFP
F = FBLN5
R = RGE-FBLN5
B = β3 integrin
D = D119A-β3 integrin

Figure 8

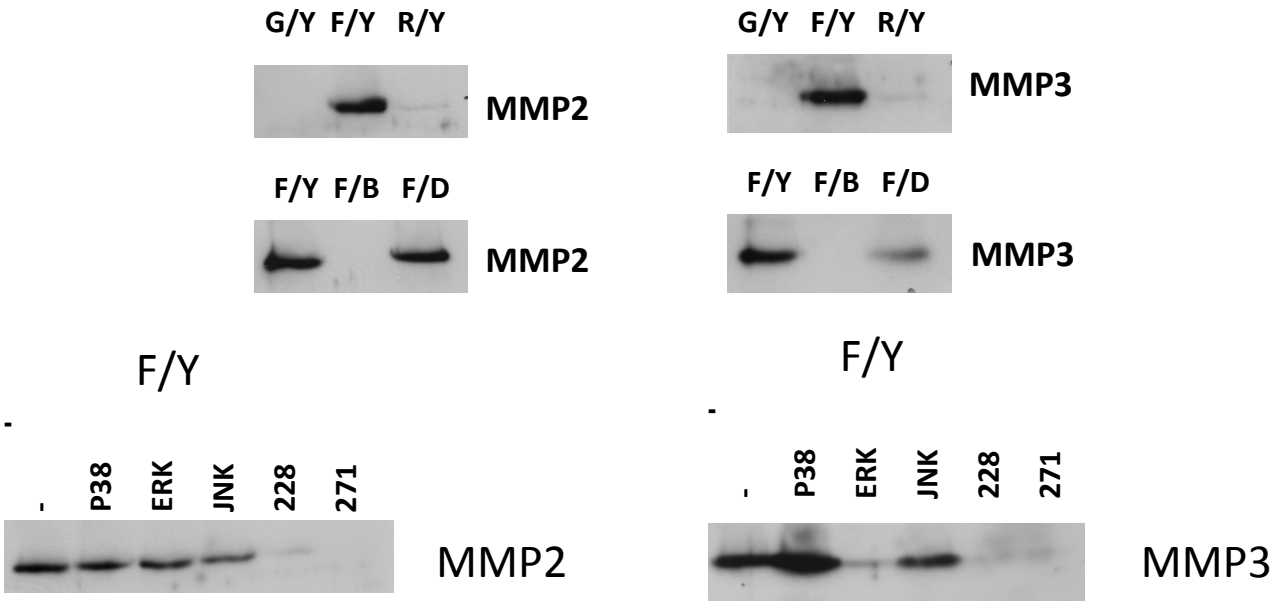
FBLN5 Regulates MMP Expression in NMuMG Cells *via* MAP Kinase-, Calcium-, and Src-Dependent Mechanisms



PKI = mixture of ERK1/2, p38 MAPK, and JNK inhibitors
Bapta = Calcium chealtor
PP2 = Src inhibitor

Figure 9

FBLN5 Induction of MMP Expression is RGD-dependent and Coupled to Activation of ERK and FAK/Pyk2: *Role of β3 Integrin in Suppressing FBLN5 Coupling to MMP Expression*



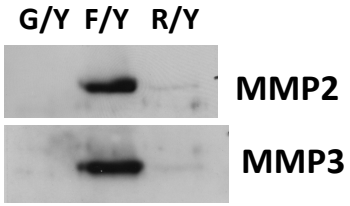
P38 = p38 MAPK inhibitor
ERK = ERK1/2 inhibitor
JNK = JNK inhibitor
228 = FAK inhibitor
271 = FAK & Pyk2 inhibitor

G = GFP
Y = YFP
F = FBLN5
R = RGE-FBLN5
B = β3 integrin
D = D119A-β3 integrin

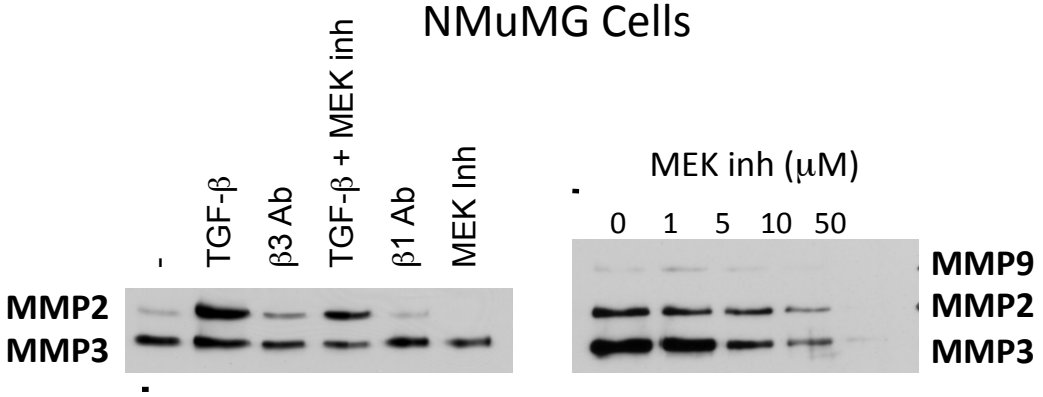
Figure 10

FBLN5 Induces MMP Expression *via* a β1 Integrin-Dependent Pathway in Normal NMuMG Cells

FBLN5 Requires Integrin Ligation to Induce MMP Expression

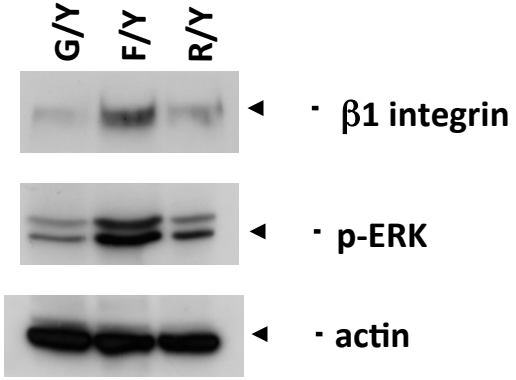


FBLN5 Induces MMP Expression *via* a β1 Integrin & ERK1/2-dependent Pathway in F/Y-Expressing NMuMG Cells



F/Y NMuMG Cells

β1 Integrin Couples FBLN5 to ERK1/2 Activation

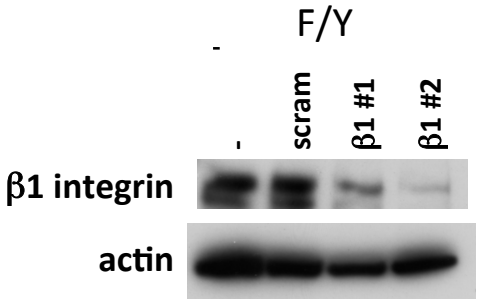


G = GFP
Y = YFP
F = FBLN5
β3 Ab = Neutralizing β3 integrin antibodies
β1 Ab = Neutralizing β1integrin antibodies
P-ERK = phosphorylated/activated ERK1/2

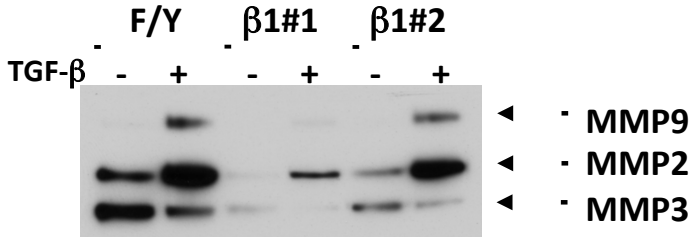
Figure 11

β1 Integrin-depletion Suppresses FBLN5- and TGF-β-mediated MMP Expression in NMuMG Cells

Monitoring the Extent of β1 Integrin-deficiency in Using Two Independent shRNAs



β1 Integrin-deficiency Uncouples FBLN5 and TGF-β From MMP Expression



Y = YFP
F = FBLN5
Scram = nonsilencing shRNA
β1 #1 = β1 integrin shRNA#1
β1 #2 = β1 integrin shRNA#2

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EMT in Tumor Metastasis: A Method to the Madness

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Keywords

Epithelial-mesenchymal transitions; metastasis; invasion; tumor microenvironment

Were it not for the ability of carcinoma cells to metastasize and colonize distant organs, all solid tumors would present medically as a group of chronic but manageable diseases. There has been significant progress in the understanding of how cancer cells acquire five of the six essential hallmarks proposed for their transformation (1). Unfortunately, it still remains unclear as to how and when cancer cell acquire the ability to metastasize – *i.e.*, the sixth and final hallmark which is responsible for more than 90 percent of the cancer related mortality (1). However, it has long been recognized that the dissemination of cancer is not simply a random dispersion of cells, but instead represents an ordered and systematic method to this madness. Indeed, epithelial-mesenchymal transition (EMT) is one such method that has been proposed to initiate the metastasis of carcinoma cells (2).

EMT was first recognized as a conserved embryonic and developmental process that facilitates the dispersion cells that ultimately lead to the generation of distinct tissue types (3). In undergoing EMT, cells lose their epithelial properties, while acquiring mesenchymal properties that enable transitioned cells to migrate to predetermined destinations (4). The idea that a similar process is reactivated during tumor progression and other pathologies, including wound healing, tissue regeneration and organ fibrosis, has gained significant ground and acceptance in recent years. Indeed, this fact is readily apparent in the sheer number of publications on this topic, and in the number of EMT-focused sessions and dedicated meetings that have grown exponentially in last few years. It is now widely accepted that EMT plays an important role during tumor progression and confers certain fundamental abilities to cancer cells that are essential for tumor metastasis. These include the ability to migrate, invade and resist anoikis (5,6).

The precise contribution of EMT to tumor metastasis is still a subject of considerable debate in the scientific literature (7). Recent reports of EMT in in-vivo animal models and human studies (8–11), to a certain degree softened the arguments for lack of concrete in-vivo evidence. However, convincing demonstration of a true phenotypic switch is still yet to come. The other dismissive argument that EMT is simply reflective of genomic instability in

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cancer cells is also fading in light of more and more studies reporting EMT that occurs in normal epithelial cells from various organs in response to injury (8,10,12).

Reports of EMT conferring resistance to certain class of drugs and therapeutic modalities, and correlation of EMT gene signatures with poor outcomes have been described (13–15). These observations, together with the recent finding that EMT may confer stem cell like properties to resulting mesenchymal cells (16) have highlighted the clinical relevance of this process. Consequently, several groups both in the industry and academia are actively pursuing the discovery of novel molecules to target EMT (17).

Recently, Kalluri and Weinberg proposed to classify EMT into three distinct subtypes based on the biological context in which they occur (4). This new terminology was not available at the time the reviews for this special supplement were accepted for publication, and as such, this classification is not used herein. With the exception of the review by Micalizzi et al., the other articles have predominantly discussed what now could be referred to as type III EMT in the new classification system – *i.e.*, EMT in the context of tumor progression. In contrast, the article by Micalizzi et al., describes the regulators of developmental EMT, which now is called type I EMT in the new classification scheme, and discusses the transcriptional reactivation of type I EMT in the context of type III EMT. Particularly interesting is the discussion of their own work investigating the role of two new players, Six 1 and Six 4, in the EMT of mouse mammary tumors. Radaelli et al., provide a very elegant historical perspective by discussing some of the early descriptions of EMT in mouse tumors, some of which date as far back as the year 1854. They also presented an interesting comparison of EMT in mouse and human pathologies. A very comprehensive review of the regulatory pathways implicated in TGF-β-induced EMT in normal and malignant cells of breast is provided in the article by Wendt et al. lastly, van Zijl et al review the evidence for EMT in hepatocellular carcinoma and discussed its implications for the treatment of these tumors.

Pathways and molecules that distinguish EMT in tumor progression from other two biological contexts are far from clear. However, any effort to identify context-specific signals should consider the physiological state of the epithelium in which EMT is taking place – *i.e.*, whether it transpires in normal, transformed, or injured epithelium, and how these unique epithelial states impact the functional consequences of the resulting EMT. Indeed, the vast majority of EMT studies to date have focused on assessing the functional consequences of EMT in solely altering the behaviors and functions of tumor cells, not their accompanying stromal components. Given the dramatic changes that take place during EMT, it is wholly reasonable to expect EMT to also elicit powerful alterations within tumor microenvironments, as well as to target the activities and behaviors of various stromal supporting cells. Therefore, the implications of EMT on the interactions of tumor cells with their accompanying stromal and microenvironmental components clearly need to be explored in the future studies.

Acknowledgments

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Papers of special note have been highlighted as either of interest (*) or of considerable interest (**) to readers

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Mechanisms of Epithelial-Mesenchymal Transition by TGF-β

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Summary

The formation of epithelial cell barriers results from the defined spatiotemporal differentiation of stem cells into a specialized and polarized epithelium, a process termed mesenchymal-epithelial transition. The reverse process, epithelial-mesenchymal transition (EMT), is a metastable process that enables polarized epithelial cells acquire a motile fibroblastoid phenotype. Physiological EMT also plays an essential role in promoting tissue healing, remodeling, or repair in response to a variety of pathological insults. On the other hand, pathophysiological EMT is a critical step in mediating the acquisition of metastatic phenotypes by localized carcinomas. Although metastasis clearly is the most lethal aspect of cancer, our knowledge of the molecular events that govern its development, including those underlying EMT, remain relatively undefined. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that oversees and directs all aspects of cell development, differentiation, and homeostasis, as well as suppresses their uncontrolled proliferation and transformation. Quite dichotomously, tumorigenesis subverts the tumor suppressing function of TGF-β, and in doing so, converts TGF-β to a tumor promoter that stimulates pathophysiological EMT and metastasis. It therefore stands to reason that determining how TGF-β induces EMT in developing neoplasms will enable science and medicine to produce novel pharmacological agents capable of preventing its ability to do so, thereby improving the clinical course of cancer patients. Here we review the cellular, molecular, and microenvironmental mechanisms used by TGF-β to mediate its stimulation of EMT in normal and malignant cells.

Keywords

Epithelial-mesenchymal Transition; Metastasis; Signal Transduction; Transforming growth factor-β; Tumor Microenvironment

1. INTRODUCTION

The epithelium is comprised of highly specialized and diverse cells that play critical roles in nearly all biological processes [1,2]. Indeed, epithelial cells serve as protective barriers that line both the outer (*i.e.*, skin) and inner (*i.e.*, airways, gastrointestinal tract, etc.) body cavities, as well as behave as secretory and glandular tissues. In addition, epithelial cell function varies widely between tissues and ranges from nutrient absorption in the intestines to gaseous exchange in the lungs to lactogenesis in the mammary gland. Equally important is the role of the epithelium in providing the first line of defense against exterior insults and infections, while simultaneously enabling the exchange of vital nutrients needed to maintain tissue homeostasis. The fidelity and function of the epithelium is maintained through its

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continual renewal and repair, and as such, it perhaps is not surprising to learn that the majority (*i.e.*, ~90%; [3]) of cancers arise in cells derived from epithelial origins. Thus, it is imperative that science and medicine uncover the sequence of events that enable specialized and polarized epithelial cells to dedifferentiate along a tumorigenic pathway that terminates in their acquisition of metastatic phenotypes.

Recent evidence has linked the development of tissue fibrosis and cancer metastasis to the inappropriate reactivation of epithelial-mesenchymal transition (EMT), which is the process whereby immotile, polarized epithelial cells transition into highly motile, apolar fibroblastoid-like cells (Figure 1; [1,2,4-6]). Indeed, EMT is a normal physiological process essential for proper embryogenesis and tissue morphogenesis, particularly for the formation of the mesoderm, neural crest, cardiac valve, and secondary palate [1,2,7]. With respect to adult tissues, EMT also is engaged in wounded epithelia to facilitate their healing, remodeling, and repair in response to tissue damage. Thus, fully differentiated epithelial cells harbor a dormant embryonic transcriptional EMT program that can be reinitiated in response to a variety of specific environmental cues and signals, one of which is the pleiotropic cytokine, transforming growth factor-β (TGF-β). Interestingly, these same cellular and morphological features are observed in cells undergoing pathophysiological EMT, which underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis, and chronic fibrotic degenerative disorders of the lung, liver, and kidney [1,2,4-6,8,9]. Along these lines, aberrant reinitiation of EMT also engenders the acquisition of invasive and metastatic phenotypes in developing and progressing carcinomas, leading to their dissemination and colonization of distant organ sites suitable to support their metastatic growth. A commonality of physiological and pathophysiological EMT is their ability to be induced by TGF-β, which now is recognized as a master regulator of this transdifferentiation process.

TGF-β is a ubiquitously expressed and multifunctional cytokine that not only regulates EMT, but also oversees the development, differentiation, and survival of essentially all cell types and tissues [10-13]. TGF-β also is a powerful suppressor of cell growth and proliferation, particularly in cells of epithelial, endothelial, and hematopoietic origins [10-13]. Quite dichotomously, aberrations in the TGF-β signaling system regularly take place during tumorigenesis and elicit resistance to its anti-proliferative activities, contributing to the formation of human neoplasms. Upon being liberated from the cytostatic activities of TGF-β, cancer cells proliferate, invade, and metastasize beyond their tissue of origin when stimulated by TGF-β. How TGF-β suppresses these processes in normal epithelial cells is unclear, as is how TGF-β promotes these processes in their malignant counterparts. Despite the continued uncertainty of the molecular events associated with the diametric activities of TGF-β, it is absolutely clear that this cytokine stimulates the two deadliest aspects of cancer, namely cell invasion and metastasis. Moreover, recent studies indicate that acquisition of metastatic phenotypes by carcinoma cells is critically dependent upon their ability to undergo EMT [4-6,8,14]. Indeed, TGF-β stimulation of EMT was demonstrated originally by Miettinen *et al* [15] who observed normal mammary epithelial cells (MECs) to acquire fibroblastoid phenotypes in response to TGF-β. In addition, TGF-β3-deficient mice develop cleft palate due to defective palatogenesis associated with aberrant EMT [16]. Similar inactivation of TGF-β2 function impairs endocardial cushion development in chick hearts due to their absence of Slug expression and its ability to activate EMT [17]. Finally, Smad3-deficiency affords protection against EMT-driven retinal [18,19] and renal [20] fibrosis in mice. Thus, these and other seminal studies have clearly established TGF-β as a master regulator of EMT. This review focuses on the myriad of evidence supporting this designation for TGF-β, particularly the cellular, molecular, and microenvironmental mechanisms that underlie the ability of TGF-β to induce EMT in normal and malignant cells.

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2. TGF-β SIGNALING & EMT

The general mechanisms whereby TGF-β activates responsive cells and regulates their behavior is depicted in Figures 2 and 3. As shown, transmembrane signaling by TGF-β commences *via* its binding to three high-affinity receptors, namely the TGF-β type I (TβR-I), type II (TβR-II), and type III (TβR-III or betaglycan). When and where it is expressed, TβR-III clearly is the most abundant TGF-β receptor on the cell surface where it functions as an accessory receptor that binds and presents TGF-β to its signaling receptors, TβR-I and TβR-II, both of which possess intrinsic Ser/Thr protein kinase activity in their cytoplasmic domains [11,12,21-23]. The binding of TGF-β to TβR-II enables the recruitment and activation of TβR-I, leading to its induction of canonical Smad2/3-dependent signaling. Once activated, Smad2/3 form heterocomplexes with Smad4 and translocate into the nucleus where they regulate the cell type-specific expression of TGF-β-responsive genes [11,12,21-23]. It is interesting to note that the variety of cell responses exhibited in response to TGF-β are governed primarily by the cell type-specific expression of various Smad2/3-interacting transcription factors (*e.g.*, AP-1 and Forkhead family members, Stats, etc. [11,22]), as well as their association with additional transcriptional activators or repressors [11,12,21-23]. Moreover, the amplitude and duration of Smad2/3 signaling is modulated by several mechanisms, including the expression of *(i)* adapter and/or anchoring proteins SARA [24], Hgs [25], and Dab2 [26] that enable Smad2/3 phosphorylation by TβR-I, and *(ii)* the inhibitory Smad, Smad7, which prevents the phosphorylation of Smad2/3 [27-29] and induces the degradation of TGF-β receptors [30,31]. In addition, the inhibitory functions of Smad7 are regulated by its interaction with STRAP [32], which potentiates the anti-TGF-β activity of Smad7, and by its association either with AMSH2 [33] or Arkadia [34-36], both of which negate the anti-TGF-β activity of Smad7. As alluded to above, the activation of Smad2/3 by TGF-β represents the canonical TGF-β signaling system, which is shown diagrammatically in Figure 3.

Also depicted in Figure 3 is the coupling of TGF-β to a variety of noncanonical signaling systems, including *(i)* the MAP kinases ERK1/ERK2, p38 MAPK, and JNK; *(ii)* the growth and survival kinases PI3K, AKT/PKB, and mTOR; and *(iii)* the small GTP-binding proteins Ras, RhoA, Rac1, and Cdc42 [37-45]. In addition, TGF-β typically represses NF-κB activity in normal epithelial cells [46,47], but readily activates this transcription factor in their malignant counterparts [47-51]. More recently, TGF-β has been shown to activate a number of protein tyrosine kinases (PTKs), including FAK [52,53], Src [43-45,54], and Abl [55,56], which results in the inappropriate amplification of noncanonical TGF-β signaling in mesenchymal or dedifferentiated epithelial cells. Moreover, imbalances in the activation status of canonical and noncanonical TGF-β signaling systems may very well underlie the ability of TGF-β to induce EMT in normal and malignant cells. The importance of canonical and noncanonical TGF-β signaling systems to promote physiological and pathophysiological EMT is presented in greater detail below.

3. DEFINING EMT

The phenomenon of EMT is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells. An inherent characteristic or hallmark of EMT, including that stimulated by TGF-β, is the dramatic phenotypic change in epithelial cell morphology [4-6,8,14]. Typically, fully differentiated epithelium manifests as a single layer of polarized epithelial cells comprised of well-defined apical and basolateral surfaces, as well as a clearly demarcated actin cytoskeleton arranged into discrete “cobblestones” that reflect regions of concentrated actin fibers at cell-cell junctions. In response to the initiation of EMT, cell-cell junctions disassemble and filamentous actin undergoes a dramatic redistribution to form prominent stress fibers, which is tracked experimentally *via* the use of

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a fluorescently-labeled mushroom toxin, phalloidin. The combined effect of these various cell biological activities is a loss of epithelial cell polarity (Figure 1).

Examining the biochemical and molecular alterations in cell-cell junction formation and dissolution has enabled science and medicine to garner a more complete assessment of the events underlying EMT. Indeed, a number of recent examinations have elucidated a variety of molecular complexes and scaffolds that govern the development of cell-cell junctions, including tight junctions, adherens junctions, and desmosomes [5]. Not surprisingly, a series of coordinated and dynamic processes underlie formation of these macromolecular complexes during the development and maintenance of the epithelium, while changes in the expression and localization of junctional proteins constitute useful measures to track the progression of EMT. For instance, tight junctions are formed by the actions of the transmembrane proteins, claudins, occludins, and JAMs (**J**unctional **A**dhesion **M**olecules), which are linked to the actin cytoskeleton *via* the scaffold proteins ZO-1, −2, −3 [57,58]. Moreover, following their formation, tight junctions and their constituents play essential roles in regulating the biology, homeostasis, and architecture of epithelial cells, and in preventing the initiation of EMT and tumorigenesis [59]. In contrast, the initiation of EMT induces a drastic modulation of tight junction localization in epithelial cells [15,38]. For instance, the function of Par6 (partitioning-defective 6), which governs the formation of tight junctions, the establishment of apical-basolateral polarity, and the initiation of polarized cell migration [60], is compromised by its physical interaction with TβR-I and subsequent phosphorylation by TβR-II in epithelial cells stimulated with TGF-β [61]. Once phosphorylated, Par6 recruits and interacts with E3 ubiquitin ligase, Smurf1, which ubiquitinates the small GTPase, RhoA, leading to its degradation and subsequent dissolution of tight junctions during EMT stimulated by TGF-β [62]. The importance of Par6 to EMT induced by TGF-β is highlighted by the ability of TβR-II-resistant Par6 mutants (*i.e.*, S345A-Par6) to prevent MECs from undergoing EMT in response to TGF-β [61].

Unlike tight junctions, adherens junctions consist of transmembrane E-cadherin (**E**pithelial-cadherin) proteins that are linked to the actin cytoskeleton by α- and β-catenins [63]. TGF-β stimulation of EMT represses E-cadherin transcription (*see below*), as well as disrupts its localization at the plasma membrane in part *via* diminished activation of the small GTPase, Rac1 [62]. The net effect of altered E-cadherin function during EMT is the dissolution of adherens junctions. In addition, the loss of cell-cell contacts parallels the development of prominent actin filaments and the appearance of fibroblastoid-like phenotypes in transitioning epithelial cells, processes requiring the activation of RhoA by TGF-β [64,65]. The mechanisms underlying TGF-β regulation of adherens junction expression and function are discussed below.

4. EMT, TGF-β, & CELL MICROENVIRONMENTS

Maintaining homeostasis within cell microenvironments is essential to alleviating disease development in humans, particularly cancer. Tumor development has been likened to that of dysfunctional miniature organs that house a mixture of malignant and normal cells, including fibroblasts, endothelial, and immune cells [66]. It also is important to remember that the growth and progression of tumors are not inherent properties of the cancer cells themselves, but instead are dictated in large part by a delicate balance between positive and negative proliferative signals produced by diverse cell types within tumor microenvironments. Indeed, alterations within tumor microenvironments can either suppress or promote cancer progression in a manner that mirrors the acquisition of oncogenic signaling by TGF-β in developing neoplasms. Biologically, TGF-β is a master inhibitor of cell cycle progression; however, this cytokine also functions as a master regulator of ECM production, deposition, and remodeling, all of which are essential processes during EMT.

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Along these lines, recent evidence has shown that TGF-β stimulation of cancer progression proceeds in part *via* its reprogramming of cell microenvironments, particularly by its ability to target the behaviors of neighboring endothelial cells (ECs) and fibroblasts. Moreover, ECs and fibroblasts typically respond to TGF-β by synthesizing and secreting numerous cytokines, growth factors, and ECM components capable of driving the progression of tumors from indolent to aggressive states [67,68]. A vital component of normal and malignant cell microenvironments is the ECM, which functions as *(i)* a gel-like structural scaffold for cells comprised of polysaccharides and fibrous proteins, including collagen, fibronectin, and elastin; and *(ii)* a molecular sensor that monitors, detects, and responds rapidly to physiological and pathophysiological changes within cell microenvironments. Indeed, under physiological conditions, the ECM serves as a storage reservoir that sequesters numerous growth factors and cytokines that can be rapidly released in response to ECM perturbations or insults, thereby circumventing the need for *de novo* protein synthesis to elicit biological behaviors [69]. Thus, the microenvironment of epithelial cells plays a critical role in maintaining their polarization and differentiation, processes that are disrupted temporarily during physiological EMT and its modification of epithelial cell microenvironments. In contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis. Table 1 identifies numerous EMT-associated genes whose expression is regulated by TGF-β, and readers desiring more in depth discussions of the activities and functions of these genes in governing EMT and epithelial cell biology are directed to several recent reviews [1,2,4-6]. In the following sections, we highlight many of the mechanisms that underlie the ability of TGF-β to induce EMT and its associated alterations within the microenvironments of transdifferentiating cells.

4.1. Matrix Metalloproteinases

Matrix **m**etallo**p**roteinases (MMPs) comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, and of wound healing and cell growth. MMPs also possess the ability to degrade nearly all ECM and basement membrane components, as well as the ability to promote the development and progression human malignancies [70,71]. Along these lines, TGF-β enhances the tumorigenicity and invasiveness of breast cancer cells by inducing their expression of MMPs 2 and 9 [72,73], which is consistent with the general importance of upregulated MMP expression in mediating the acquisition invasive phenotypes in several cancers [74]. Indeed, aberrant MMP expression (*e.g.*, MMP-7 or matrilysin) facilitates the development of mammary fibrosis and desmoplasia, which increase tumor rigidity and the selection, expansion, and dissemination of metastatic cells [75,76]. Similarly, upregulated MMP-3 expression is sufficient to induce lung and mammary fibrosis [77,78], and to stimulate EMT in carcinomas [79]. Thus, elucidating the connections between aberrant MMP expression and the development of fibrosis and/or EMT will offer important clues as to how EMT promotes cancer progression. For instance, does pathophysiologic EMT solely mediate the acquisition of invasive phenotypes by developing carcinomas, or does this event simply reflect the transdifferentiation of a subset of carcinoma cells into tumor supporting stroma cells (*e.g.*, myofibroblasts) [80]? Indeed, tumor-associated myofibroblasts upregulate their production and secretion of TGF-β, which may serve in establishing a positive feedback loop that drives the selection and expansion of metastatic carcinoma cells [81-83]. Collectively, these findings point to the need for additional studies to fully address these questions, particularly since the expression and activity of MMPs alters the expression of E-cadherin, Snail, vimentin, and TGF-β in a manner consistent with the induction of EMT [79].

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4.2. Neuronal Cell Adhesion Molecule

Neuronal **c**ell **a**dhesion **m**olecule (NCAM) is a member of the immunoglobulin superfamily and has been implicated as a mediator of tumor progression and metastasis [84]. Recently, TGF-β stimulation of EMT was observed to induce NCAM expression in a manner correlated with downregulated expression E-cadherin [85]. Functionally, upregulated expression of NCAM during EMT facilitates the formation of β1 integrin-containing focal adhesion complexes [85]. Interestingly, the extracellular domain of NCAM is cleaved proteolytically by MMP-28 (epilysin), which also induces EMT through its ability activate latent TGF-β complexes from inactive ECM depots [86]. In addition, MMP-28 expression also is upregulated in a EMT-dependent manner in wounded epithelial cells, and in metastatic breast cancer cells [87]. Thus, future studies need to determine the physiological and pathophysiological connections between NCAM, MMP-28, and TGF-β during the initiation of EMT in normal and malignant epithelial cells.

4.3. Urokinase Plasminogen Activator

Urokinase **p**lasminogen **a**ctivator (uPA) is a serine protease whose elevated expression in human cancer correlates with advanced disease sates and poor clinical outcomes, presumably through its ability to promote cancer cell invasion and metastasis [88,89]. Accordingly, uPA expression is essential for breast and ovarian cancer metastasis in mice [90,91], and for hypoxia-induced EMT in breast cancer cells *via* uPA receptor-mediated activation of AKT and Rac1 [92]. TGF-β is a potent inducer of uPA expression, yet the role of this event in mediating EMT and metastasis stimulated by TGF-β remains to be elucidated fully. Recently, the activation of JNK1/2 was shown to be essential for TGF-β stimulation of uPA expression and EMT [93], which is consistent with the notion that noncanonical TGF-β signaling promotes its oncogenic activities in epithelial cells.

4.4. Plasminogen Activator Inhibitor-1

Plasminogen **a**ctivator **i**nhibitor-1 (PAI-1) is an antagonist of tissue-type plasminogen activator (tPA) and uPA, as well as a physical interactor of the ECM ligand, vitronectin [94,95]. tPA and uPA both activate the serine protease activity of plasminogens (or plasmins), resulting in the degradation of blood plasma proteins, such as fibrin and von Willebrand factor, and of ECM proteins, such as fibronectin, thrombospondin, and laminin [95]. Through its ability to inhibit tPA and uPA, PAI-1 prevents the activation of intravascular and cell-associated plasminogen, and as such, impedes the breakdown of blood clots and ECM proteins necessary to enable carcinoma cells to undergo invasion and extravasation reactions during metastasis [95].

TGF-β is principal player involved in stimulating PAI-1 transcription in part *via* activation of p53, which binds and stabilizes PAI-1 transcripts [96,97]. Quite dichotomously, overexpression of PAI-1 has been observed to reduce the migration and invasion of breast and ovarian cancers [94,98]; however, PAI-1 polymorphisms or its aberrantly elevated expression also have been associated with a poor prognosis and the increased risk of metastasis in breast cancer patients [99]. Thus, the precise mechanisms underlying the dynamic relationship between PAI-1, plasminogen, and TGF-β regulatory loops, as well as their impact on cancer cell motility, remain an active and important topic of investigation.

4.5. Collagen

Collagen is an abundant ECM molecule that assembles into tensilely strong fibers that provide mechanical support to tissues. The major types of collagen, types I-IV, are distributed differentially in specific tissues of the body. For instance, collagen IV is a major component of the basal lamina, a specialized component of the basement membrane in the

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mammary gland. Invading breast cancer cells must degrade collagen IV to migrate into surrounding tissue. Interestingly, Endo180 is a cell surface receptor that promotes the uptake of collagen for its degradation intracellularly. Moreover, Endo180 expression is *(i)* elevated significantly in highly invasive breast cancer cells; *(ii)* induced transcriptionally by TGF-β stimulation in breast cancer cells; and *(iii)* reduced the collagen content and enhanced the growth of mammary tumors produced in mice [100]. In addition, TGF-β also governs collagen function by upregulating the expression of MMP-2 and other collagenases in normal and malignant MECs, leading to their enhanced migration and invasion [72,73,101].

4.6. Fibronectin

Fibronectin is a large and critical ECM glycoprotein whose elevated production by cancer cells classically is associated with the acquisition of EMT, and more recently, with the development of the metastatic niche [67]. TGF-β is a potent inducer of fibronectin production and deposition into the ECM [102], where it binds integrins and regulates cell adhesion and motility. The synthesis and secretion of fibronectin into the ECM is primarily mediated by fibroblasts, and by epithelial cells induced to undergo EMT (Table 1; [103]). With respect to the latter, nontumorigenic EpH4 MECs engineered to express oncogenic Ras (*i.e.*, EpRas cells) significantly upregulate their expression of fibronectin and its receptor, α5β1 integrin when stimulated with TGF-β [104]. More importantly, administration of neutralizing α5 integrin antibodies to TGF-β treated EpRas cells inhibited their migration and induced a significant apoptotic response [104]. Thus, the synthesis and deposition of fibronectin, coupled with changes in expression and activation of integrins (*see below*), clearly represent an important mechanism that enables TGF-β to stimulate invasive migration during EMT.

4.7. Cadherin Switching

A phenotypic hallmark of EMT is its ability to downregulate the expression and function of E-cadherin, which is critical in mediating epithelial cell integrity and cell-cell adhesion [105]. Reduced E-cadherin expression in developing and progressing carcinomas takes places through several mechanisms that function en masse to promote cancer cell invasion [5]. For instance, E-cadherin can be inactivated by genetic mutations, and humans harboring these E-cadherin mutations have significantly increased risk of developing cancer [106]. In addition, epigenetic silencing of the E-cadherin *(CDH1)* promoter *via* hypermethylation of its 5′ CpG island also enhances the development of carcinomas [107]. Along these lines, TGF-β stimulation of EMT also represses the synthesis of E-cadherin transcripts in large part *via* its ability to induce the expression of the Snail/ZEB family of basic helix-loop-helix transcription factors, including that of Snail1, ZEB1, Snail2/Slug, Twist, and ZEB2/SIP1 [105,108-110]. Although the relative contribution of canonical and noncanonical TGF-β signaling in mediating transcriptional activation of these E-cadherin repressors remains to be determined definitely, recent evidence suggests that these events do take place in a cell type-specific manner in response to TGF-β. For example, activation of Smad2/3 by TGF-β in MECs induces their expression of the nuclear high mobility group A2 (HMGA2), which promotes EMT by stimulating the expression of Snail1, Snail2/Slug, and Twist, and by inhibiting the expression of ID2 (inhibitor of differentiation 2) [111]. In addition, while the functional consequences of diminished E-cadherin expression on the behavior of transitioning epithelial cells is well established, recent studies have determined that these same cells also exhibit upregulated expression of N-cadherin (*i.e.*, **N**euronal-cadherin) [65], an event linked to elevated cell motility and poor clinical outcomes in cancer patients [112-114]. At present, the necessity of increased N-cadherin expression in mediating EMT, particularly that stimulated by TGF-β, remains to be clarified. Indeed, we [45] and others [115,116] recently established murine 4T1 breast cancer cells as a model of advanced stage breast cancer whose increased malignancy is governed by TGF-β. Interestingly, while 4T1

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cells undergo EMT and downregulate E-cadherin when stimulated by TGF-β [44,45], these cells fail to express and/or elevate their expression of N-cadherin during EMT initiated by TGF-β (M.K. Wendt and W.P. Schiemann, *unpublished observation*). Thus, future studies aimed at determining the exact nature of N-cadherin in promoting the acquisition of EMT and metastatic phenotypes clearly are warranted.

4.8. Vimentin

The intermediate filament protein vimentin is expressed in all primitive cell types, but not in their differentiated counterparts. In light of its role as a master regulator of EMT, it perhaps is not surprising to learn that TGF-β stimulation of EMT reactivates vimentin expression in dedifferentiating epithelial cells, an event that serves as a canonical marker for detecting fully transitioned epithelial cells and their acquisition of fibroblastoid-like phenotype [117].

4.9. α-Smooth Muscle Actin

A major component of contractile microfilaments is α-smooth muscle actin (α-SMA), which also serves as a canonical marker for detecting fibroblasts/mesenchymal cells, particularly myofibroblasts. Indeed, during its induction of EMT, TGF-β stimulates α-SMA expression in transitioning epithelial cells [118], an event associated with increased tumor invasion and decreased patient survival rates [119].

5. TRANSMEMBERANE & MEMBRANE PROXIMAL PROTEIN COMPLEXES THAT IMPACT TGF-β SIGNALING & EMT

Recent evidence suggests that cell surface signaling receptors, such as receptor tyrosine kinases (RTKs) and G-protein-coupled receptors, do not function in isolation and instead require accessory signaling inputs that arise from interacting receptors and signaling modules. As shown in Figure 3, the function and behavior of TGF-β receptors also are modulated *via* their association with an ever expanding array of receptor-interacting molecules and scaffolding proteins. Included in this growing list of TGF-β receptor regulators are members of the integrin superfamily of heterodimeric transmembrane adhesion receptors, which function as direct physical conduits that link the ECM to the cytoskeleton of the cell [120,121]. Integrin signaling commences upon their clustering and subsequent stimulation of the Ser/Thr protein kinase ILK (**i**ntegrin-**l**inked **k**inase), as well as members of the Src family of PTKs and FAK (**f**ocal **a**dhesion **k**inase), leading to the activation of a vast array of downstream effectors, including members of the MAP kinase family of protein kinases, members of the Ras/Rho family of small GTPases, and members of the PI3K and AKT signaling axes [120-124]. Integrins also regulate cell behavior through their ability to form complexes with RTKs [125,126]. For instance, β1 integrins form FAK-dependent complexes with the receptors for EGF, PDGF, and HGF [126,127], and in doing so, enables growth factor-mediated induction of cell migration and invasion [126]. Interestingly, the scaffolding function of FAK is independent of its PTK activity, but does require its N-terminal FERM (**F**AK **E**zrin **R**adixin **M**oesin) domain and C-terminal FAT (**F**ocal **A**dhesion **T**argeting) domain to bind RTKs and β1 integrins, respectively [126]. Lastly, the establishment of EMT phenotypes in cultured cells, as well as the development of late stage cancers and their acquisition of invasive and metastatic phenotypes both have been linked to dramatic changes in the expression and localization of integrins in epithelial cells [104,128].

In addition to its regulation of cell cycle progression, TGF-β also figures prominently in mediating ECM remodeling and repair *via* its ability to regulate integrin expression [129-131]. Moreover, αvβ6 and αvβ8 integrin ligation promotes the activation of TGF-β1 and TGF-β3 from inactive ECM depots [132-137], which regulates alveolar development,

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wound closure, fibrosis, and EMT [130,132,138-140]. In addition, epidermal transgenic expression of α6β4 integrin also elicits elevated development of metastatic papillomas and carcinomas in a chemical carcinogenesis model of skin cancer. Importantly, the tumorigenicity associated with α6β4 integrin expression was linked to its ability to uncouple TGF-β from activating Smad2/3 and preventing cell cycle progression [141]. Similar reciprocity between integrins and TGF-β is observed in cancers of the prostate, whose metastasis to bone is stimulated by TGF-β and its induction of α2β1 integrin, which binds to bone-derived type I collagen [142]. Thus, the ability of TGF-β to stimulate cancer progression and metastasis requires an intricate interplay between signals arising from TGF-β receptors and those initiated by integrins. Accordingly, integrins have been found to associate with TGF-β receptors and play a critical function in coupling TGF-β to activation of its noncanonical effectors, and to its induction of EMT. For instance, neutralizing β1 integrin antibodies abrogated the ability of TGF-β to activate p38 MAPK and induce EMT in MECs [39]. Similarly, hepatocellular carcinoma cells elevate their expression of α3β1 integrin in response to TGF-β, an event that enhances their motility and invasiveness [143]. Moreover, administering laminin-5 together with TGF-β stimulated hepatocellular carcinoma cells to undergo EMT in an α3 integrin-dependent manner [144], further demonstrating the necessity of integrins to cooperate with TGF-β to induce EMT and invasion in transitioning cells.

We also described the functional cooperation between integrins and TGF-β in promoting EMT, as well as in stimulating the development and progression of mammary tumors. For instance, we found the expression and activity of αvβ3 integrin and its downstream effector Src to be essential for TGF-β stimulation of MEC proliferation, invasion, and EMT [43-45]. In addition, transgenic expression of αvβ3 integrin not only negated the cytostatic response of normal MECs to TGF-β, but also enhanced its stimulation of MEC invasion and p38 MAPK activation. Importantly, inactivation of either αvβ3 integrin or Src function abolished the ability of TGF-β to stimulate EMT and invasion in normal and malignant MECs [43,44]. Mechanistically, β3 integrin interacts physically with TβR-II, leading to its *(i)* phosphorylation on Y284 by Src; *(ii)* interaction with Grb2 and Shc at phosphorylated Y284; *(iii)* activation of p38 MAPK; and *(iv)* stimulation of EMT and invasive migration in normal and malignant MECs [44]. Along these lines, the growth and metastasis of breast cancer cells in mice absolutely required TβR-II to be phosphorylated on Y284, a phosphotransferase reaction that disrupts the delicate balance between canonical and noncanonical TGF-β signaling inputs activated during mammary tumorigenesis [45]. In addition to its ability to promote pulmonary metastasis stimulated by TGF-β [45], αvβ3 integrin expression also directs breast cancer cell metastasis to bone [145,146] and lung [146] in part through a TGF-β-dependent pathway. Collectively, these findings suggest that pharmacological targeting of noncanonical TGF-β effectors, particularly αvβ3 integrin, Src, and p38 MAPK, may prove efficacious in treating metastatic breast cancers.

Besides integrins, a growing number of intracellular proteins also have been shown to interact with and regulate the activity of TGF-β receptors. For instance, two members of the focal adhesion complex, namely FAK and its downstream effector p130Cas (p130**C**rk-**a**ssociated **s**ubstrate), both influence the cellular response to TGF-β through dramatically different mechanisms. Indeed, TGF-β stimulates FAK and its relative PYK2 during EMT [147], leading to the activation of JNK and the subsequent upregulation α-SMA in fibroblasts [148]. In addition, FAK activation in hepatocytes is necessary for the transcription of mesenchymal and invasive gene expression profiles, as well as for the delocalization of E-cadherin from the plasma membrane [149]. Finally, we recently established FAK as a molecular scaffold that facilitates the formation of oncogenic β3 integrin:TβR-II complexes and their activation of Src and interaction with Grb2 (M.K. Wendt and W.P. Schiemann, *unpublished observation*). Moreover, the ability of FAK to

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form these signaling complexes is essential for TGF-β stimulation of p38 MAPK in breast cancer cells, as well as for their induction of EMT and metastasis stimulated by TGF-β (M.K. Wendt and W.P. Schiemann, *unpublished observation*; [45]). Thus, the aberrant recruitment of FAK to TGF-β receptors readily influences the oncogenic conversion of TGF-β from a tumor suppressor to a tumor promoter, including its stimulation of pathophysiological EMT in carcinoma cells.

In stark contrast to FAK, the incorporation of p130Cas into active TGF-β receptor complexes alters the coupling of TGF-β to the canonical Smad2/3 pathway. Indeed, the activation and phosphorylation of p130Cas following cellular adhesion to ECM matrices led to its association and inactivation of Smad3, and to diminished cytostatic activity of TGF-β [150]. Similarly, we find that rendering malignant, metastatic MECs deficient in p130Cas enhances Smad2/3 activation by TGF-β, but fails to alter its coupling to p38 MAPK; however, this same cellular condition selectively inhibited breast cancer metastasis only in cells that possessed heightened TGF-β signaling (M.K. Wendt and W.P. Schiemann, *unpublished observation*), suggesting that p130Cas acts as a molecular integrator of canonical Smad2/3 signaling when confronted with elevated oncogenic behavior mediated by the receptors for TGF-β or EGF [151].

Recently, the regulation of TGF-β signaling has been shown to be modulated by two additional adapter proteins that localize to focal adhesions, namely Hic5 and Disabled-2 (Dab2). Indeed, Hic5 is a member of the paxillin superfamily and, like paxillin, functions as an adapter protein at focal adhesions [152], as well as resides in the nucleus where functions as a transcriptional coactivator in regulating gene expression induced by the androgen [153] and glucocorticoid [154,155]. Moreover, Hic5 expression is low in quiescent MECs, but is induced rapidly *via* a RhoA/ROCK-dependent pathway following administration of TGF-β [152]. In addition, uncoupling Hic5 from TGF-β regulation prevents its induction of EMT in normal MECs [156]. Thus, Hic5 plays an essential role in coupling TGF-β receptors to activation of RhoA/ROCK and, consequently, to the induction of EMT. Along these lines, Dab2 was identified originally as an ovarian tumor suppressor gene [157,158] that regulates the actin cytoskeletal architecture during cell migration and adhesion [159]. More recently, Prunier *et al* [160] established Dab2 as a novel gene target of TGF-β in MECs undergoing EMT in part *via* its ability to *(i)* associate with TGF-β receptor complexes [26]; *(ii)* promote Smad2/3 activation by TGF-β receptors [26]; and *(iii)* stimulate the activation of TAK1 and JNK, which induced fibronectin expression and enhanced cell motility [161]. Along these lines, TGF-β stimulates Dab2 expression in MECs undergoing EMT, which promotes the formation of Dab2:β1 integrin complexes and their activation of FAK [160]. Importantly, measures capable of disrupting Dab2 function prevents EMT stimulated by TGF-β, as well as promotes its ability to induce apoptosis in MECs. Although the molecular mechanisms underlying the ability of TGF-β to stimulate Dab2 expression remains to be defined, these studies do provide interesting insights into the connections that govern alterations in cell survival and morphology regulated by TGF-β.

Finally, two laboratories recently identified a novel collaboration between signaling molecules activated by TNF-α and those activated by TGF-β. Indeed, both studies demonstrated the ability of TGF-β to induce the physical association of its receptors with that of TRAF6 (**T**NF **r**eceptor-**a**ssociated **f**actor 6) [162,163], leading to K63-linked polyubiquitination and activation of TAK1 and its subsequent stimulation of p38 MAPK and JNK. Moreover, whereas TRAF6-deficiency had no effect on the coupling of TGF-β to Smad2/3, this same cellular condition uncoupled TGF-β from activation of MAP kinases and prevented this cytokine from inducing EMT in normal MECs [163]. Taken together, these studies reinforce the notion that imbalances in the TGF-β signaling system that favor its activation of noncanonical effectors over that of its canonical Smads are crucial to its

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induction of EMT in normal and malignant epithelial cells. These findings also point to the need for additional studies to define precisely how these aberrant protein complexes and modules impact the epithelial cell response to TGF-β, and how science and medicine can better target these effector molecules that promote oncogenic signaling and EMT initiation by TGF-β.

6. SIGNALING SYSTEMS INVOLVED IN EMT STIMULATED BY TGF-β

Transmembrane signaling by TGF-β traditionally is associated with its activation of Smad2/3 and their ability to alter the transcription of TGF-β-responsive genes, which clearly play an important role in mediating the ability of TGF-β to induce EMT, tumor formation, and cancer cell metastasis [164]. The necessity of Smads 2 and 3 for TGF-β stimulation of EMT has been reviewed extensively in the scientific literature, and readers desiring a more in depth description of Smad2/3 function in regulating EMT in normal and malignant cells are directed to several recent reviews [4,5,11]. As alluded to above, the enhanced coupling of TGF-β to its noncanonical effectors figures prominently in mediating its biological and pathological behaviors, particularly its ability to induce EMT and cancer cell metastasis. Table 2 lists a variety of noncanonical effectors targeted by TGF-β during its activation of EMT, while the role of these signaling molecules during epithelial cell EMT induced by TGF-β is discussed below.

6.1. Rho Family of Small GTPases

The Rho family of small GTPases is comprised of RhoA, Rac1, and Cdc42, which regulate the formation of stress fibers, lamellipodia, or filopodia, respectively [165,166]. Indeed, Rac1 is an established inducer of cell-cell adhesions in epithelial cells [167], which contrasts sharply with the ability of RhoA to dissolve these adhesive complexes to facilitate times of cell migration [62]. Given the importance of these small GTPases in overseeing cell adhesion, morphology, and migration, it is fitting to find that these effectors are intimately involved in EMT stimulated by TGF-β. For instance, the activation of RhoA by TGF-β enables MECs to undergo EMT, while measures capable of inhibiting RhoA function or that of its downstream effector, $p160^{ROCK}$, uncouple TGF-β from EMT in MECs [65]. Moreover, RhoA activation also is essential for TGF-β stimulation of α-SMA expression in renal epithelial cells undergoing EMT [118]; however, completion of this same cellular event in lens epithelial cells requires signaling inputs from both RhoA/ROCK and Smad2/3 [168]. Taken together, these studies point to the overall importance of noncanonical TGF-β signaling, particularly that induced by RhoA/ROCK, to induce EMT in epithelial cells.

6.2. PI3K/AKT

The tumor suppressing activity of TGF-β not only reflects its ability to induce cytostasis, but also its propensity to activate apoptosis in epithelial cells [10,11,13,169]. Interestingly, the ability of TGF-β to stimulate apoptosis frequently is subverted during tumorigenesis, leading to enhanced cancer cell survival *via* activation of the PI3K and AKT signaling systems by TGF-β. Indeed, administration of PI3K inhibitors to MECs inhibits their activation of AKT and ability to undergo EMT in response to TGF-β [38]. The activation of AKT by TGF-β can transpire directly *via* TGF-β receptors or indirectly *via* the transactivation of EGF [170] and PDGF [171] receptors, which induces the expression of genes operant in mediating cancer cell EMT, metastasis, and survival. In addition to altering gene expression profiles, AKT also regulates mRNA translation when impacting the response of epithelial cells to TGF-β. For instance, TGF-β stimulation of EMT in MECs is accompanied by an increase in cell size and protein content, both of which correlate with the rapid activation of mTOR (**m**ammalian **t**arget **o**f **r**apamycin) in transitioning MECs [40]. Somewhat unexpectedly, administering the mTOR inhibitor, rapamycin, to MECs failed to affect their acquisition of

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an EMT morphology in response to TGF-β; however, this same cellular condition completely prevented the ability of TGF-β to increase MEC size and protein production, as well as inhibited their migration and invasion [40]. Taken together, these findings highlight an important bifurcation in the TGF-β signaling system that dissociates the ability of TGF-β to alter cell morphology from its ability to elevate cell motility. Future studies need to identify the transcriptional and translational objectives targeted by TGF-β, as well as determine their relative contribution to oncogenic signaling stimulated by TGF-β in normal and malignant cells.

6.3. Integrin-linked Kinase (ILK)

In addition to their stimulation of PTKs, the ECM engagement of β1 and β3 integrins also activates the Ser/Thr protein kinase ILK and its ability to mediate the *(i)* stimulation of MAP kinases, PI3K/AKT, and small GTPases; and *(ii)* the inhibition of GSK3β [172-174]. Accordingly, targeting ILK expression to mouse mammary glands elicited a hyperplastic reaction that progressed to full-blown breast cancer in part *via* constitutive activation of ERK1/2 and AKT, which inactivated GSK3β [175]. Elevated ILK expression is associated with the acquisition of EMT phenotypes by MECs, including reductions in their expression of E-cadherin and adhesion, as well as increases in their formation of actin stress fibers and invasion [176]. ILK also participates in EMT stimulated by TGF-β by coupling this cytokine to its activation of AKT [177], and to its elevated expression of MMP-2 and uPA [91]. Collectively, these findings suggest that ILK may function analogously to FAK in mediating oncogenic signaling by TGF-β, and as such, suggest that ILK interfaces integrin signaling with that stimulated by TGF-β in epithelial cells undergoing EMT.

6.4. NF-κB

NF-κB is a principal player involved in regulating the production of proinflammatory cytokines [178], and in stimulating tumor growth, vascularization, survival, and invasion [178]. In addition, NF-κB activity was observed to be essential in mediating the ability of Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β [48,179]. Along these lines, NF-κB activity also associates with several hallmarks of EMT, including downregulated E-cadherin expression and upregulated expression of vimentin [180]. It is interesting to note that TGF-β typically represses NF-κB activity in normal epithelial cells [47,181,182], but readily induces the activation of this transcription factor in their malignant counterparts [47,182]. Recently, we demonstrated that the activation of NF-κB by TGF-β transpires *via* the aberrant formation of a TAB1:xIAP:TAK1:IKKβ signaling module that only materializes in malignant MECs, or in normal MECs following their induction of EMT by TGF-β [47]. Functionally, the formation of TAB1:xIAP:TAK1:IKKβ complexes is essential for TGF-β stimulation of *(i)* Cox-2 expression and its induction of EMT and invasion in normal and malignant MECs [47,182]; and *(ii)* mammary tumor growth in immunocompetent and immunocompromised mice [47], suggesting a potentially important role of NF-κB in regulating innate immunity by TGF-β. Collectively, these findings demonstrate the role of NF-κB in supporting the development of oncogenic signaling by TGF-β in normal and malignant cells, particularly its ability to drive the growth, metastasis, and EMT of tumors in response to TGF-β.

6.5. MAP Kinases

Members of the MAP kinase family of protein kinases, which includes ERK1/2, JNKs, and p38 MAPKs, all have been implicated in mediating EMT and metastasis stimulated by TGF-β [39,183,184]. For instance, pharmacological inhibition of ERK1/2 in MECs uncouples TGF-β from inducing EMT and its associated formation of stress fibers and delocalization of ZO-1 and E-cadherin [183]. Similarly, inactivation of JNK also prevents the ability of TGF-β to stimulate the morphological and transcriptional changes that drive EMT in epithelial

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cells [93,161]. Indeed, the activation of JNK by TGF-β induces fibronectin expression during EMT, and during fibroproliferative disorders that may progress to carcinoma [185]. Along these lines, collagen I and other ECM proteins can promote EMT *via* their activation of PI3K, Rac1, and JNK [186]; however, while it remains to be determined whether TGF-β is intimately involved in this ECM-dependent induction of EMT, it seems likely that the ability of TGF-β to stimulate the synthesis and secretion of ECM components is reciprocated by the ability of the ECM to establish paracrine and autocrine TGF-β signaling loops that perpetuate EMT in normal and malignant epithelial cells.

Besides its ability to activate ERK1/2 and JNK, TGF-β also stimulates p38 MAPK during its induction of EMT in normal and malignant cells [39]. Interestingly, the activation of p38 MAPK by TGF-β requires the expression and activity of either β1 or β3 integrins [39,43,44]. Indeed, we established the necessity of β3 integrin to form oncogenic signaling complexes with TβR-II, resulting in its phosphorylation on Y284 by Src [43,44]. Once phosphorylated, Y284 functions as a SH2-binding site that coordinates the recruitment of either ShcA or Grb2, as well as their subsequent activation of p38 MAPK [44]. Most importantly, pharmacologic or genetic inactivation of this oncogenic signaling axis prevented TGF-β from stimulating the growth and pulmonary metastasis of breast cancers produced in mice [45]. Finally, the activation of p38 MAPK not only induces EMT, but it also stimulates the expression of prometastatic genes, particularly TβR-II and MMPs 2 and 9 [187,188], which collectively points to the importance of inappropriate p38 MAPK activation in mediating the conversion of TGF-β from a tumor suppressor to a tumor promoter.

7. MECHANISMS OF GENE REGULATION BY TGF-β

The importance of aberrant genetic and epigenetic events in promoting tumorigenesis is highlighted by the consistent and repeated finding that cancer cells that have lost their ability to regulate various rate-limiting steps that normally suppress malignant development. These untoward events typically are associated with the *(i)* mutational activation of oncogenes, *(ii)* mutational inactivation of tumor suppressor genes, or *(iii)* amplified or silenced expression of genes coupled to the development of cancer hallmarks [189]. Although many of the signaling systems and genes targeted by TGF-β during its activation of EMT have been discussed above, the succeeding sections focus on the transcriptional mechanisms that orchestrate its transitioning of epithelial cells into their mesenchymal counterparts (Figure 3).

7.1. Nuclear Factors

The Snail family of transcription factors are master regulators of EMT and include *(i)* SNAI1 (Snail) and SNAI2 (Slug); *(ii)* two ZEB factors, ZEB1 and ZEB2 (SIP1); and *(iii)* FOXC2 [110,190]. Indeed, the binding of Snail to conserved E-box sequences present in E-cadherin promoter is classically associated with EMT and the repression of E-cadherin expression, as well as that of the aforementioned cell polarity genes, occludin and claudin [191]. The essential function of various Snail family members in mediating EMT and cancer metastasis have been extensively reviewed, and as such, readers desiring a more in-depth description of their functions and behaviors in governing EMT are directed several recent reviews [109,190]. Besides Snail family members, emerging evidence also implicates dysregulated Myc expression in promoting the ability of epithelial cells to undergo EMT in response to TGF-β. Indeed, the tumor suppressing activity of TGF-β is intimately linked to its ability to rapidly repress Myc expression in epithelial cells [11,13]. Accordingly, uncoupling TGF-β from regulation of Myc expression is a common occurrence in developing carcinomas, resulting in their insensitivity to cytostasis mediated by TGF-β [192,193]. Somewhat unexpectedly, Myc recently was observed to function cooperatively with Smad4 to induce Snail expression during TGF-β stimulation of EMT in MECs [194].

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Taken together, these findings suggest the Myc functions as a molecular detector that enables epithelial cells to sense TGF-β as a mediator of cytostasis or EMT.

7.2. STAT3

Signal **t**ransducer and **a**ctivator of **t**ranscription 3 (STAT3) is a critical component of cell survival and proliferative responses, and its inappropriate activation can endow this transcription factor with oncogene-like properties in developing and progressing neoplasms [195]. A recent study has suggested that TGF-β couples to STAT3 phosphorylation and activation *via* a PKA-dependent mechanism [196]. Moreover, STAT3 activation by TGF-β is necessary for its ability to induce apoptosis and EMT [196], and to stimulate the invasion and metastasis of Smad4-deficient pancreatic cancer cells [197]. In addition, carcinoma cells that overexpressed EGFR readily acquired EMT phenotypes when stimulated with EGF, a cellular reaction that required EGF/EGFR to activate STAT3 and its subsequent upregulation of TWIST [198]. Thus, while several studies have shown EGF to cooperate with TGF-β in mediating tumorigenesis, the extent to which this tumor-and EMT-promoting alliance requires STAT3 remains to be determined definitively.

7.3. Estrogen Receptor-α

Aberrant repression of the nuclear hormone receptor, estrogen receptor-α ER-α) has long been recognized as a major event that promotes the development and progression of mammary tumors, as well as significantly worsens the clinical prognosis of patients with metastatic breast cancer [199,200]. In addition to its prominent role in regulating mammary gland development and homeostasis, ER-α also prevents the ability of malignant MECs to acquire EMT and metastatic phenotypes, doing so *via* its stimulation of MTA3 (metastasis tumor antigen 3) expression, which in turn represses the expression of Snail [201]. Thus, inactivation or loss of ER-α in MECs promotes their EMT and invasion by allowing for their expression of Snail. Somewhat surprisingly, constitutive Snail expression in breast cancer cells was observed to inhibit ER-α expression [202], leading to enhanced invasion of these ER-α-deficient breast cancer cells. It is interesting to note that physiological actions of estrogen in mammary tissues typically oppose those of TGF-β. Accordingly, inactivation of ER-α signaling led to elevated expression of components of the TGF-β signaling system and, presumably, to enhanced EMT in breast cancer cells [202]. Thus, Snail appears to function as a novel molecular sensor that integrates the opposing cellular functions of ER-α and TGF-β, particularly their ability to inhibit and stimulate EMT, respectively.

7.4. TGF-β, microRNAs, & EMT

A number of recent studies have established microRNAs as important players that participate in cell and tissue development, as well as control cell proliferation and motility through their ability to repress mRNA translation, or to induce mRNA degradation [203-206]. These studies also have shown that a single microRNA can repress the translation of multiple transcripts, and as such, dysregulated expression of a single microRNA, either positively or negatively, could initiate a cascade of gene silencing events capable of eliciting disease development in humans, including cancer. Accordingly, aberrant regulation of several microRNAs (or miRs) is observed in human cancers (see [207]), especially in those of the breast, which can in fact be subtyped based on their differential expression of various microRNAs [205,208]. Along these lines, microRNAs also play a prominent role in regulating the expression of EMT-related genes. For instance, members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2 (SIP1), which as mentioned above function in repressing the expression of E-cadherin [209-211]. Indeed, miR-200 family member expression marks epithelial cells that express E-cadherin and not vimentin, as well as identifies cancer cells that are poorly motile [212]. With respect to EMT and its regulation by TGF-β, a recent study established that this

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cytokine downregulates the expression of microRNA-200 family members and miR-205, which promotes ZEB1 and ZEB2 expression and their initiation of EMT [211]. In addition, these same microRNAs are frequently downregulated in invasive human breast cancer cells that exhibit a mesenchymal-like morphology [211]. Somewhat surprisingly, elevated ZEB1 expression also was found to repress that of miR-41 and miR-200c, both of which belong to the miR-200 family and whose absence establishes a negative feedback loop that stabilizes the acquisition of EMT phenotypes in epithelial cells [213].

In contrast to the miR-200 family of microRNAs, metastatic breast cancers were found to preferentially upregulate their expression of miR-10b, which promotes the invasion and metastasis of malignant MECs both *in vitro* and *in vivo* [214]. Mechanistically, Twist was observed to induce miR-10b expression that results in the *(i)* diminished translation of HoxD10 transcripts, and *(ii)* induction of the prometastatic gene, RhoC [214]. More recently, administration of TGF-β to normal MECs induced miR-155 expression *via* a Smad4-dependent mechanism, an event that elicited EMT in cytokine-stimulated MECs [215]. Once expressed, miR-155 abrogated MEC expression of RhoA and prevented their ability to undergo EMT in response to TGF-β [215]. Similar overexpression of miR-21 also is observed in human cancers and results in the repression of the tumor suppressor, tropomyosin-1 [216,217]. The net effect of these events is the enhanced ability of breast cancer cells to grow in an anchorage-independent fashion [218], and to resist apoptotic stimuli in part *via* upregulated expression of the survival factor, Bcl-2 [216-218]. As above, the ability of TGF-β to induce EMT has been linked to its induction of miR-21 [219], which enhances cancer cell motility and invasive migration by downregulating tropomyosin expression [220-222].

Taken together, these findings suggest that the ability of TGF-β to govern microRNA expression plays an important role in dictating whether this cytokine propagates tumor suppressing or promoting signals to responsive cells; they also suggest that the development of chemotherapeutic agents capable of targeting microRNAs may function in “normalizing” carcinoma cells and, consequently, rendering them insensitive to the oncogenic activities of TGF-β.

7.5. DNA Hypermethylation

DNA hypermethylation is well established in its ability to aberrantly silence the expression of tumor suppressor genes in developing and progressing carcinomas [107]. Importantly, epigenetic silencing of the E-cadherin promoter *via* hypermethylation leads to morphological and differential gene expression profiles indicative of EMT phenotypes [107,223]. Besides silencing of the E-cadherin promoter, EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to promote expanded DNA hypermethylation. Indeed, Roberts *et al* [224] observed the loss of p16INH4a expression to depress that of the polycomb genes, EZH2 and SUZ12, which collectively enhance DNA hypermethylation and the generation of MECs locked into a perpetual plastic state. Interestingly, the repression of E-cadherin expression during EMT appears to function as a prerequisite for directed gene hypermethylation during the development and progression of mammary tumorigenesis [225]. Moreover, hypermethylation of the E-cadherin promoter served to mark stable EMT in Ras-transformed MECs that was induced by serum *versus* a transient EMT induced in these same MECs by TGF-β [225]. Clearly, additional investigations are warranted to further our understanding of the linkages between TGF-β and DNA hypermethylation in mediating EMT in normal and malignant cells. Indeed, upregulated ZEB1 expression and its ability to induce EMT is tightly correlated with the loss of E-cadherin expression in cultured epithelial cells, and in metastatic carcinoma cells *in vivo* [226]. Based on these findings, it is tempting to speculate that initiation of EMT results in *(i)* the expression of Snail family members that collectively function in repressing

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that of E-cadherin, and *(ii)* the subsequent recruitment of DNA methyltransferases that potentiate and stabilize the EMT phenotype.

8. FUTURE PERSPECTIVE

Embryogenesis and its associated EMT creates progenitor cells that ultimately give rise to every cell- and tissue-type within mature organisms. For instance, EMT underlying gastrulation results in the generation of the mesoderm, which subsequently develops along distinct differentiation pathways that elicit the production of muscle, bone, and connective tissues [7]. Similarly, a single mammary stem cell can give rise to both the outer myoepithelial and inner luminal layers that comprise the branched structure of these glands[227-229]. These and other studies suggest an important link between physiologic EMT and the generation and maintenance of stem cells, of which both phenomena require signaling inputs elicited by the TGF-β signaling system [230]. Given the parallels between physiologic and pathophysiologic EMT, it is fitting to find that the inappropriate reactivation of EMT in malignant tissues also promotes the selection and expansion of cancer stem cells. For instance, aggressive and poorly differentiated breast cancer and glioma cells exhibit gene signatures characteristic of stem cells [231]. In addition, TGF-β stimulation of EMT in human and mouse MECs established a population of transitioning cells that possessed stem cell-like properties [232,233], suggesting that EMT induced by TGF-β promotes “stemness.” Along these lines, inactivation of TGF-β signaling in cancer stem cells induced a mesenchymal-epithelial transition that reestablished a more epithelial-like morphology in aggressive cancer cells [234]. Thus, these intriguing findings suggest that the ability of TGF-β to stimulate the selection and expansion of stem cell-like progenitors in post-EMT epithelial cells may represent the molecular crux that endows TGF-β with oncogenic activity. Clinically, these findings also suggest that the development of chemoresistance may reflect the induction of EMT and its expansion of cancer stem cells by TGF-β. If correct, then the studies reviewed herein offer important insights into how science and medicine may one day target the TGF-β signaling system and its coupling to EMT in order to *(i)* regulate the behaviors and activities of normal and cancer stem cells, and *(ii)* alleviate the devastating effects of TGF-β in promoting the acquisition of invasive and metastatic phenotypes in human cancers.

Executive Summary

Defining EMT

- EMT is defined by the morphologic and genetic transition of epithelial cells to fibroblastoid- or mesenchymal-like cells.
- The major cell-cell junctions include tight junctions, adherens junctions, and desmosomes.
- Tight junctions are composed of claudins, occludins, and JAMs, which are linked to the actin cytoskeleton *via* ZO-1, −2, and −3.
- During EMT, Par6 recruits the E3 ubiquitin ligase, Smurf1, which ubiquitinates RhoA, leading to its degradation and subsequent dissolution of tight junctions.
- Adherens junctions consist E-cadherin that is linked to the actin cytoskeleton by α- and β-catenins.
- EMT represses E-cadherin transcription and disrupts its localization at the plasma membrane.

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EMT, TGF-β, and Cell Microenvironments

- Tumors house a mixture of malignant and normal cells, including fibroblasts, endothelial, and infiltrating immune cells, which collectively comprise the tumor microenvironment.
- Alterations within tumor microenvironments can either suppress or promote cancer progression in a manner that mirrors the acquisition of oncogenic signaling by TGF-β.
- Transient disruption of the ECM and epithelial cell microenvironments are characteristic of physiological EMT. In contrast, chronic disruptions within carcinoma cell microenvironments elicits pathologic EMT and its ability to support cancer cell invasion and metastasis.
- MMPs comprise a large family of proteases that regulate essential steps of embryogenesis and tissue morphogenesis, wound healing, and cell growth. MMPs also degrade nearly all ECM and basement membrane components, leading to the development and progression human malignancies.
- NCAM is a member of the immunoglobulin superfamily whose expression is increased during EMT.
- The extracellular portion of NCAM is cleaved by MMP-28.
- uPA is a serine protease whose expression is elevated during EMT and associates with advanced disease states and poor clinical outcomes.
- PAI-1 antagonizes tPA and uPA; its expression also is increased during EMT and associates with advanced disease states and poor clinical outcomes.
- EMT leads to the upregulation of collagen and fibronectin, whose expression drastically alters cell microenvironments.
- Vimentin is an intermediate filament protein, while α-smooth muscle actin is a component of contractile microfilaments. Upregulated expression of both proteins are considered to be markers of fully transitioned cells.

Transmembrane and Membrane Proximal Protein Complexes the Impact TGF-β Signaling and EMT

- α and β integrin heterodimers function in linking the ECM to intracellular signaling pathways, and to the cellular cytoskeletal system.
- Integrins interact with several intracellular kinases, as well as several transmembrane RTKs.
- Integrin β1 and β3 interact with TβR-II and profoundly affect downstream signaling events stimulated by TGF-β.
- β3 integrin is upregulated dramatically during EMT induced by TGF-β.
- Interaction between αvβ3 integrin and TβR-II leads to Src-mediated phosphorylation of TβR-II at Tyr284, which binds Grb2 and promotes the activation of downstream MAP kinases.
- The ability of TGF-β to stimulate cancer progression and metastasis requires an intricate interplay between signals arising from TGF-β receptors and those initiated by integrins.
- FAK is required for EMT stimulated by TGF-β.

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- p130Cas inhibits Smad3 activity and alters cytostasis induced by TGF-β.
- Hic5 is a member of the paxillin superfamily that is induced by and required for EMT stimulated by TGF-β.
- TRAF6 interacts physically with both TβR-I and TβR-II, leading to TAK1 activation and the stimulation of p38 MAPK and JNK.

Signaling Systems Involved in EMT Stimulated by TGF-β

- Transmembrane signaling by TGF-β activates Smad2/3 and their ability to alter the transcription of TGF-β-responsive genes, which play important roles during TGF-β stimulation of cancer cell EMT and metastasis.
- Small GTPases RhoA, Rac1, and Cdc42 regulate the formation of stress fibers, lamellipodia, or filopodia, respectively, and are intimately involved in EMT stimulated by TGF-β.
- The ability of TGF-β to stimulate apoptosis frequently is subverted during tumorigenesis, leading to enhanced cancer cell survival *via* activation of the PI3K and AKT signaling systems.
- ECM engagement of β1 and β3 integrins activates the Ser/Thr protein kinase ILK and its ability to mediate the *(i)* stimulation of MAP kinases, and *(ii)* the inhibition of GSK3β, PI3K/AKT, and small GTPases.
- ILK participates in EMT stimulated by TGF-β by coupling this cytokine the activation of AKT, and to the elevated expression of MMP-2 and uPA.
- NF-κB activity enables Ras-transformed breast cancer cells to undergo EMT and colonize the lung when stimulated by TGF-β.
- Activation of NF-κB also associates with several hallmarks of EMT, including the downregulated expression of E-cadherin and upregulated expression of vimentin.
- MAP kinase family members, including ERK1/2, JNK, and p38 MAPK, have all been implicated in mediating EMT and metastasis stimulated by TGF-β.

Mechanisms of Gene Regulation by TGF-β

- Aberrant genetic and epigenetic events promote tumorigenesis by circumventing various rate-limiting cellular steps that normally suppress neoplastic development. These key steps are known as the “Hallmarks of Cancer” and include *(i)* oncogene activation; *(ii)* tumor suppressor inactivation; *(iii)* apoptosis resistance; *(iv)* angiogenesis activation; *(v)* invasion and metastasis initiation; and *(vi)* immortality acquisition.
- Snail transcription factor family members are master regulators of EMT and include *(i)* SNAI1 (Snail) and SNAI2 (Slug); *(ii)* ZEB1 and ZEB2 (SIP1); and *(iii)* FOXC2.
- Evidence implicates dysregulated Myc expression in promoting the ability of epithelial cells to undergo EMT in response to TGF-β. The tumor suppressing activity of TGF-β is intimately linked to its repression of Myc expression in epithelial cells.
- STAT3 mediates cell survival and proliferative signals, and serves as an oncogene in several human cancers.

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- TGF-β activates STAT3 *via* a PKA-dependent mechanism, leading to the induction of EMT.
- ER-α promotes mammary gland develop and homeostasis, and suppresses EMT by inducing the expression of MTA3 (metastasis tumor antigen 3), which represses the expression of Snail.
- microRNAs are essential mediators of all facets of cell and tissue development, and of cell proliferation, motility, and survival.
- Members of the miR-200 family suppress EMT by downregulating the expression of ZEB1 and ZEB2.
- Epigenetic silencing of the E-cadherin promoter *via* hypermethylation promotes the acquisition of EMT phenotypes and gene expression profiles.
- EMT and mammary tumorigenesis usurp the inactivation of p16INK4a as a means to expand aberrant DNA hypermethylation.

Redefining EMT Induced by TGF-β

- Inappropriate reactivation of EMT by TGF-β in malignant tissues promotes the selection and expansion of cancer stem and progenitor cells.
- Targeting the molecular links between TGF-β, EMT, and stemness reduces breast cancer tumorigenicity.
- The development of pharmacological agents that inhibit EMT stimulated by TGF-β may provide new avenues to manipulate the behaviors of normal and cancer stem cells, and to alleviate the acquisition of cancer metastasis.

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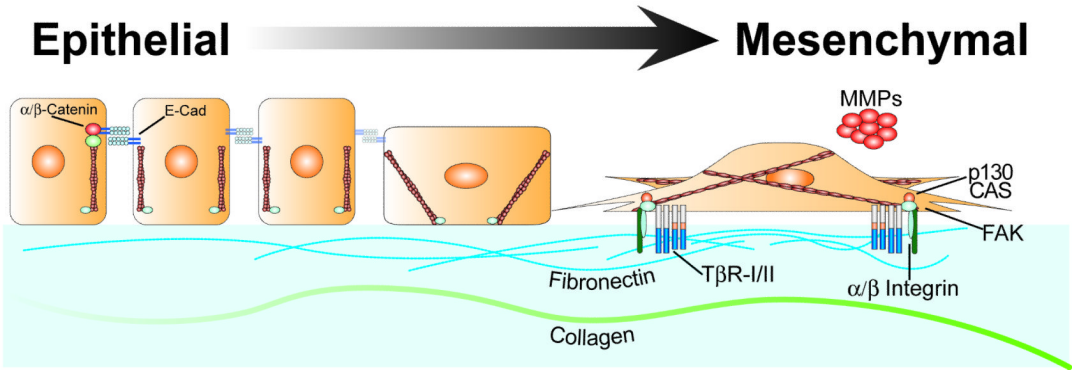

Figure 1. Epithelial Cells Transition to Mesenchymal-like Cells in Response to TGF-β

This schematic depicts polarized epithelial cells and their cuboidal structure that is maintained *via* cell-cell junctions comprised of homotypic E-cadherin molecules that are linked to the cortical actin cytoskeleton by α- and β-catenins. TGF-β stimulation of EMT during wound healing or tumor invasive migration results in the delocalization, degradation, and/or downregulation of cell-cell junctions and, consequently, a loss of epithelial integrity. In addition, the morphologic transition of epithelial cells also is supported by the simultaneous formation of actin stress fibers, the upregulation of integrins, and the activation of focal adhesion complexes. Moreover, the increased production and section of ECM proteins, such as fibronectin and collagen, coupled with the elevated expression and activation of MMPs enables transitioned fibroblastoid-like cells to exhibit invasive and motile phenotypes.

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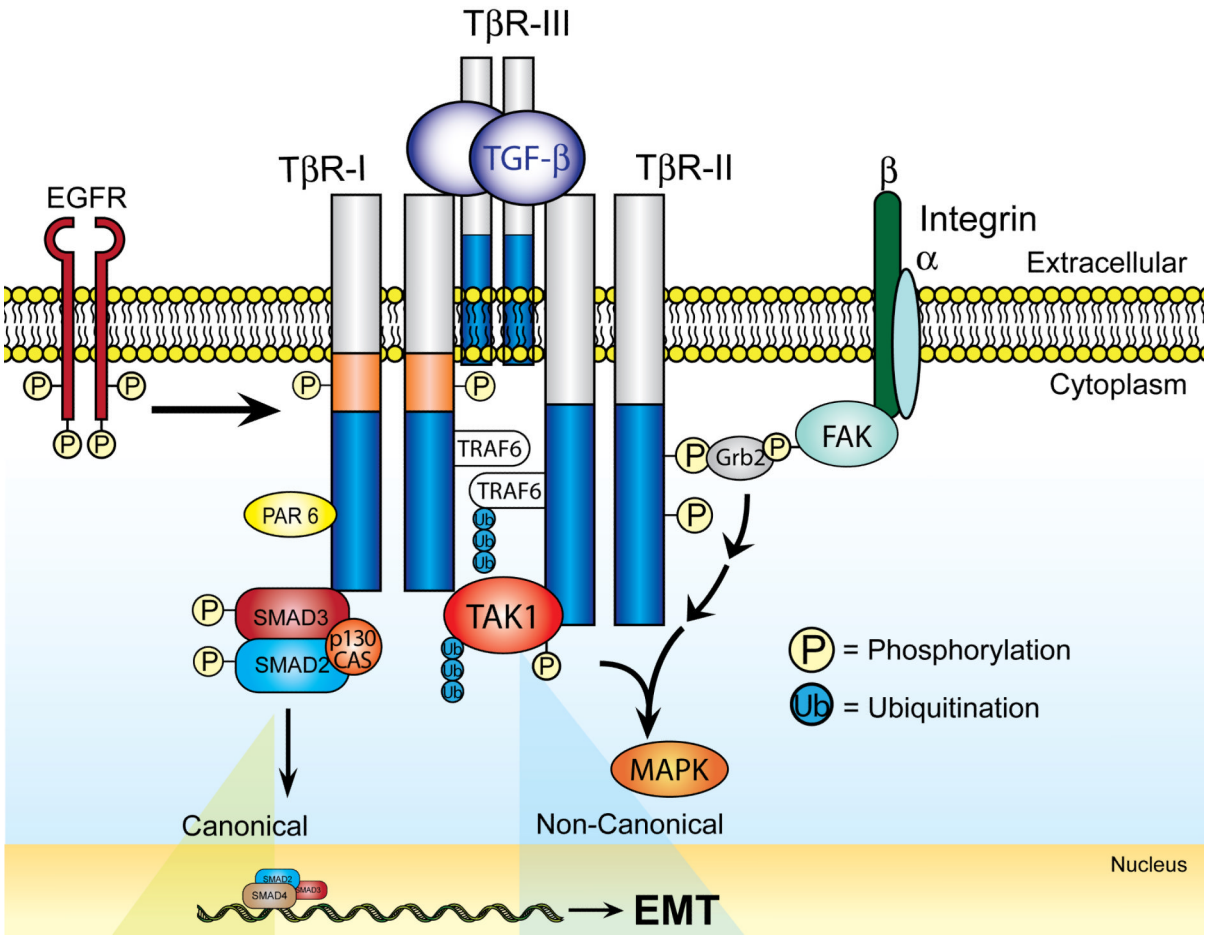

Figure 2. Differential Interactions of TGF-β Receptors with Transmembrane and Membrane Proximal Proteins Complexes Facilitate the Diversity TGF-β Signaling

β1 and β3 integrins interact physically with TβR-II [43-45]. The association of TβR-II with β3 integrin is mediated by FAK, which facilitates the binding of TβR-II to the SH2-binding protein, Grb2. In addition, TβR-II also interacts physically with EGFR (M.K. Wendt and W.P. Schiemann, *unpublished observation*), which also is activated indirectly by TGF-β through its increased synthesis and secretion of EGFR ligands. The cytoplasmic tails of both TβR-I and TβR-II interact with TRAF6, which ubiquitinates itself and the MAPKKK, TAK1. Additional interactions include the binding of p130Cas to Smad3, as well as that of PAR6 with TβR-I. Importantly, the differential composition of TGF-β receptor and scaffolding complexes directs the coupling of TGF-β to canonical and noncanonical effector activation, as well as underlies the pathophysiological conversion of TGF-β signaling and EMT in malignant epithelial cells. The biological outcomes of these various protein-protein interactions are discussed in the text.

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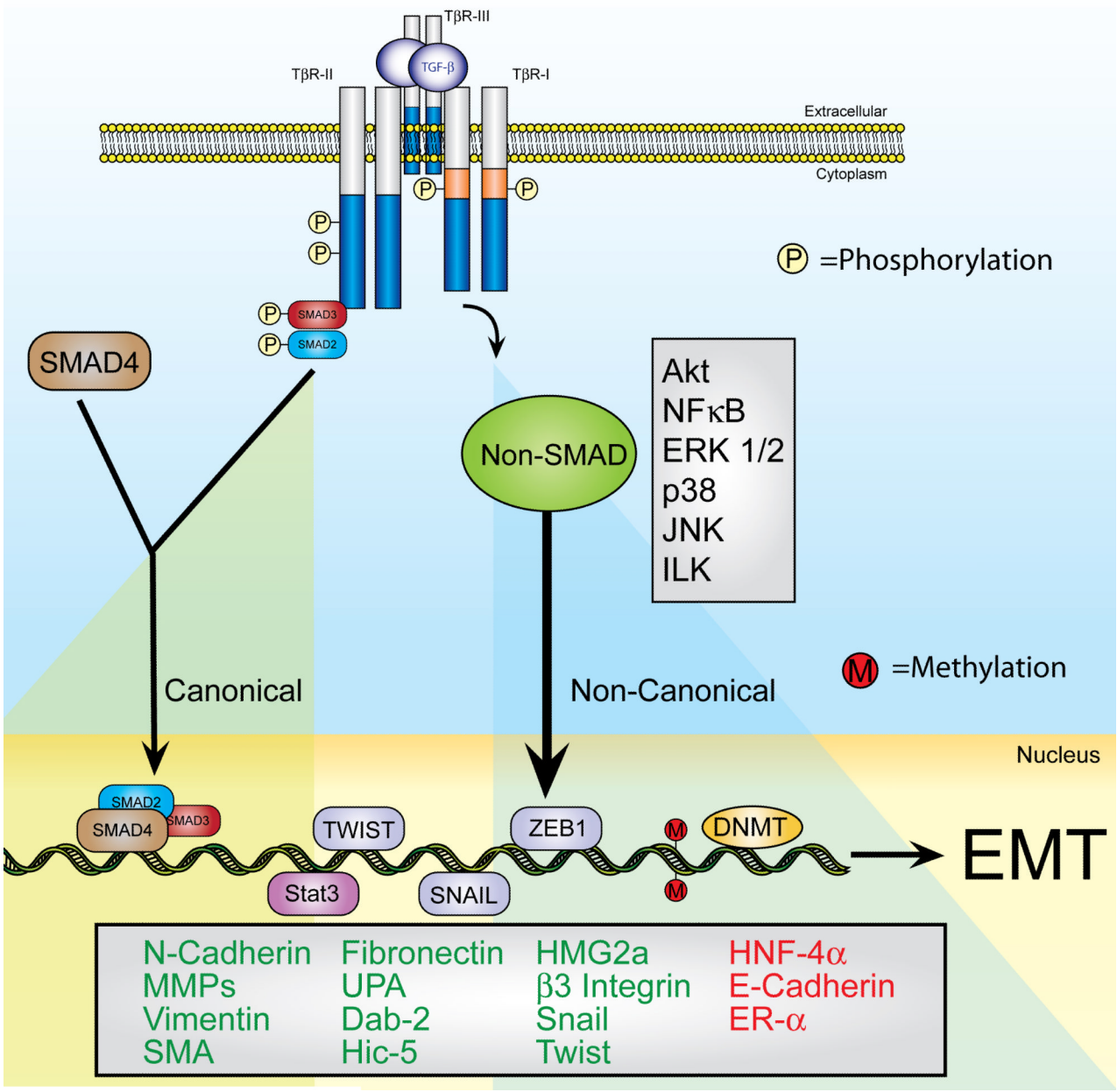

Figure 3. Diverse TGF-β Signaling Pathways Support a Complex Transcriptional Response During EMT

TGF-β stimulates epithelial cells by binding and activating two transmembrane Ser/Thr protein kinase receptors, namely TGF-β type I (TβR-I) and type II (TβR-II). Activation of these ligand:receptor ternary complexes requires TβR-II to transphosphorylate TβR-I, which phosphorylates and activates Smad2/3. Once activated, Smad2/3 form heterocomplexes with Smad4, which collectively translocate to the nucleus to mediate canonical signaling events by TGF-β (left panel). Noncanonical (right panel) TGF-β signaling takes place through its ability to stimulate various alternate signaling pathways discussed in detail herein. Activation of canonical Smad2/3 signaling results in their nuclear translocation with Smad4 and subsequent regulation of gene expression through their numerous interactions with additional transcriptional activators and repressors. Alternatively, activation of noncanonical TGF-β signaling, such as MAP kinases, small GTPases, PI3K/AKT, and NF-κB, also

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couples TGF-β to its regulation of gene expression profiles operant in mediating EMT. Finally, activation of the transcription factors belonging to the Snail family (*e.g.*, Snail, Twist, or ZEB1), or of Stat3 elicit EMT-gene expression, which ultimately promotes the prolonged induction of EMT and fibroblastoid-like phenotypes of carcinoma cells *via* DNA methylation-mediated silencing of E-cadherin expression. Altered coupling of TGF-β to its canonical and noncanonical effector pathways leads to differential gene expression patterns that ultimately contribute to the development of oncogenic signaling by TGF-β. Indeed, the initiation of oncogenic signaling by TGF-β converts its regulation of physiological EMT in normal epithelial cells to one of pathophysiologic EMT in their malignant counterparts.

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Table 1

Expression of EMT-associated Genes Targeted by TGF-β

Gene Name	Expression Change	Reference
E-cadherin	Decrease	Miettinen *et al* [15]
β3 integrin	Increase	Galliher *et al* [43]
N-cadherin	Increase	Hazan *et al* [112]
NCAM	Increase	Lehembre *et al* [85]
MMP-2	Increase	Duivenvoorden *et al* [72]
MMP-3	Increase	Farina *et al* [235]; Radisky et al [79]
MMP-9	Increase	Farina *et al* [235]; Kim *et al* [73]
Vimentin	Increase	Grunert *et al* [117]
α-Smooth Muscle Actin	Increase	Masszi *et al* [118]
Fibronectin	Increase	Ignotz *et al* [102]
Estrogen Receptor-α	Decrease	Dhasarathy *et al* [202]
Urokinase Plasminogen Activator	Increase	Farina *et al* [235]
Dab2	Increase	Hocevar *et al* [26]
Hic5	Increase	Tumbarello *et al* [156]
HMG2A	Increase	Thuault *et al* [111]

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Table 2

Signaling Pathways Activated During EMT Stimulated by TGF-β

Pathway	Reference
Smad2/3	Piek *et al* [236]
Rho family of small GTPases	Bhowmick *et al* [65]
PI3K and AKT	Bakin *et al* [38]
NF-κB	Huber *et al* [179]
ERK1/2	Xie *et al* [183]
p38 MAPK	Bhowmick *et al* [39]; Galliher and Schiemann [43-45]
JNK	Hocevar *et al* [185]
Integrin-linked kinase	Lee *et al* [177]; Lin *et al* [91]

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The TGF-β Paradox in Human Cancer: An Update

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Summary

Transforming growth factor-β (TGF-β) plays an essential role in maintaining tissue homeostasis through its ability to induce cell cycle arrest, differentiation, apoptosis, and to preserve genomic stability. Thus, TGF-β is a potent anticancer agent that prohibits the uncontrolled proliferation of epithelial, endothelial, and hematopoietic cells. Interestingly, tumorigenesis typically elicits aberrations in the TGF-β signaling pathway that engenders resistance to the cytostatic activities of TGF-β, thereby enhancing the development and progression of human malignances. Moreover, these genetic and epigenetic events conspire to convert TGF-β from a suppressor of tumor formation to a promoter of their growth, invasion, and metastasis. The dichotomous nature of TGF-β during tumorigenesis is known as the "TGF-β Paradox," which remains the most critical and mysterious question concerning the physiopathological role of this multifunctional cytokine. Here we review recent findings that directly impact our understanding of the "TGF-β Paradox" and discuss their importance to targeting the oncogenic activities of TGFβ in developing and progressing neoplasms.

Keywords

Angiogenesis; Cancer; Cell Invasion; Epithelial-mesenchymal Transition; Metastasis; Signal Transduction; Transforming growth factor-β

1. TGF-β and the Tumor Microenvironment

1.1. TGF-β and Fibroblasts

Tumor development in many respects mirrors that of an organ, albeit in a highly dysfunctional and disorganized manner. For instance, whereas normal tissue specification requires reciprocal signaling inputs from distinct cell types and matrix proteins, the phenotype of developing carcinomas is similarly dictated by the dynamic interplay between malignant cells and their accompanying stroma, which houses fibroblasts and endothelial cells (ECs), as well as a variety of infiltrating immune and progenitor cells [1,2]. Moreover, tumor reactive stroma not only plays an important role during cancer initiation and progression, but also in determining whether TGF-β suppresses or promotes tumor formation (Figure 1; [3–5]). Along these lines, TGF-β exerts its anti-tumor activities by regulating epithelial cell behavior, and by regulating that of adjacent fibroblasts, which synthesize and secrete a variety of cytokines, growth factors, and extracellular matrix (ECM) proteins that mediate tissue homeostasis and suppress cancer development. Thus, inactivating paracrine TGF-β signaling between adjacent epithelial and stromal compartments promotes cellular transformation, as well as induces the growth, survival, and motility of developing neoplasms [6,7]. For instance, rendering fibroblasts deficient in the expression of the TGF-β type II receptor (TβR-II), which manifests as

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insensitivity to TGF-β, results in the formation of prostate intraepithelial neoplasia and invasive carcinoma of the forestomach [3]. Conditional deletion of TβR-II in mammary gland fibroblasts enhanced their proliferation and abundance within abnormally developed ductal units [8]. Interestingly, grafting a mixture of TβR-II-deficient mammary fibroblasts with mammary carcinoma cells under the subrenal capsule significantly enhanced the growth and invasion of breast cancer cells. The enhanced tumorigenicity of implanted mammary carcinoma cells was not recapitulated in grafts containing TGF-β-responsive fibroblasts, which failed to synthesize and secrete the high levels of TGF-α, MSP (macrophage-stimulating protein), and HGF (hepatocyte growth factor) produced by their TβR-II-deficient counterparts [3–5,8]. Thus, TGF-β signaling in fibroblasts suppresses their activation of cancer-promoting paracrine signaling axes that target adjacent epithelial cells. Somewhat surprisingly, TβR-II-deletion in mammary carcinoma cells resulted in the activation of two tumorigenic paracrine signaling axes comprised of SDF-1:CXCR4 and CXCL5:CXCR2, which collectively function in recruiting immature $GR1^+CD11b^+$ myeloid cells to developing mammary tumors [9]. Upon their arrival within mammary tumor microenvironments, $GR1^+CD11b^+$ cells promote breast cancer cell invasion and metastasis by attenuating host tumor immunosurveillance, and by stimulating MMP expression [9]. Recently, the ability of TGF-β to induce cell cycle progression in glioma cells required initiation of autocrine PDGF-B signaling. Importantly, the proliferation promoting properties of TGF-β and Smad2/3 only occurred in glioma lacking methylation of the PDGF-B gene, suggesting that the methylation status of PDGF-B determines the oncogenic activities of TGF-β in part *via* autocrine PDGF-B signaling within tumor microenvironments [10].

Tumorigenesis often is accompanied by intense desmoplastic and fibrotic reactions, which elicit the formation of rigid tumor microenvironments that enhance the selection and expansion of metastatic cells [11,12]. Lysyl oxidases (LOX) belong to a 5 gene family of copper-dependent amine oxidases (*i.e.*, LOX, LOXL, LOXL2, LOXL3, and LOXL4) that function in cross-linking collagens to elastin in the ECM [13,14]. Mechanistically, the activation of these cross-linking reactions by LOXs secreted by fibroblasts and epithelial cells serves to increase the tensile strength and structural integrity of tissues during embryonic development and organogenesis, as well as during the maintenance of normal tissue homeostasis [13,14]. Similar to TGF-β, members of the LOX family have been associated with tumor suppression and tumor promotion. Indeed, the transformation of fibroblasts by oncogenic Ras is suppressed by LOX and its ability to bind, oxidize, and inactivate growth factors housed in cell microenvironments, which presumably contributes the loss of cyclin D1 expression observed in LOX-expressing fibroblasts [15,16]. More recently, LOX was observed to interact physically with TGFβ1 and alter its ability to stimulate Smad3 in cultured osteoblasts [17], while LOXL4 expression inhibited TGF-β stimulation of liver cancer cell invasion through synthetic basement membranes [18]. Thus, these findings implicate LOXs as potential suppressive agents within tumor microenvironments. In stark contrast, aberrant LOX activity also is associated with cancer progression, particularly the selection, expansion, and dissemination of metastatic cells [13–15,19–22]. Indeed, upregulated LOX expression is *(i)* essential for hypoxia-induced metastasis of human MDA-MB-231 breast cancer cells in mice [19]; and *(ii)* is observed most frequently in poorly differentiated, high grade mammary tumors and, consequently, predicts for increased disease recurrence and decreased patient survival [5,19]. Recently, we observed LOX expression to be induced strongly by TGF-β in normal and malignant MECs, and in mammary tumors produced in mice. Moreover, inhibiting LOX activity or degrading its metabolic byproduct, hydrogen peroxide, both antagonize the ability of TGF-β to induce the proliferation, EMT, and invasion in normal and malignant MECs. Furthermore, we find that LOX antagonism uncouples TGF-β from stimulating Src and p38 MAPK (M. Taylor and W.P. Schiemann, *unpublished findings*), whose activities are essential for mediating oncogenic signaling by TGF-β in breast cancer cells [23–25]. Along these lines, future studies need to enhance our understanding of *(i)* the role of tumor reactive fibroblasts and their production of

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TGF-β in protecting carcinoma cells from tumoricidal radiotherapies, and *(ii)* the molecular and cellular mechanisms whereby anti-TGF-β therapies selectively sensitize carcinoma cells, not their adjacent normal counterparts, to ionizing radiation treatments [26–28].

Collectively, these findings highlight the important role TGF-β plays in governing autocrine and paracrine signaling networks, and more importantly, demonstrate how disrupting the delicate balance between these systems contributes to carcinoma development and progression.

1.2. TGF-β and Immunosurveillance

In addition to its regulation of stromal fibroblasts, TGF-β present in cell microenvironments also plays an essential role in governing the delicate balance between host immunosurveillance and inflammation, which collectively can determine whether tumor development and progression is induced or inhibited [29,30]. The importance of TGF-β in regulating immune system function and homeostasis is underscored by the finding that *(i)* TGF-β1-deficient mice exhibit lethal multifocal inflammatory disease [31,32]; *(ii)* Smad3-deficient mice exhibit defects in the responsiveness and chemotaxis of their neutrophils, and their T and B cells [33]; and *(iii)* transgenic expression of truncated TβR-II specifically in T cells results in severe autoimmune reactions characterized by multifocal inflammation and autoantibody production [31]. Furthermore, T cell-specific deletion of Smad4 in mice drives T cell differentiation towards a Th2 phenotype and their elevated secretion of interleukins (ILs) 4, 5, 6, and 13 [34]. Similar to fibroblasts, the net effect of disrupting paracrine T cell signaling networks is the development of gastrointestinal carcinomas in these genetically engineered animals [34]. In addition, cancer cells typically increase their production and secretion of TGFβ into tumor microenvironments, as well as into the general circulation of cancer patients [35–37]. Abnormally elevated TGF-β concentrations also are detected within the tumor milieu in response to ECM degradation mediated by resident and recruited leukocytes –*i.e.*, monocytes/macrophages, dendritic cells, granulocytes, mast cells, T cells, and natural killer (NK) cells – that either promote or suppress tumor development in a context-specific manner [38].

1.2.1. TGF-β and Adaptive Immunity—TGF-β suppresses host immunosurveillance by inhibiting the proliferation and differentiation of NK and T cells, and by diminishing their synthesis and secretion of cytotoxic effector molecules, including interferon-γ, lymphotoxin-α, perforin/granzyme, and Fas ligand [30,39,40]. TGF-β also inhibits the tumor-targeting activities of T and NK cells through its stimulation of Tregs housed within tumor microenvironments [41]. Whereas TGF-β potently inhibits the proliferation of naïve $CD8^+$ T cells, this cytokine elicits little-to-no activity in fully differentiated $CD8^+$ T cells due to their downregulation of TβR-II. Administration of ILs 2 or 10 to differentiated $CD8^+$ T cells restores their responsiveness to TGFβ, as does expression of the co-stimulatory molecule CD28, which promotes the survival of memory/effector phenotypes in thymic and peripheral T cell populations [30,39,42]. Mechanistically, the immunosuppressive effects of TGFβ transpire in part *via* Smad3, whose phosphorylation and activation prevents the mitogenesis of $CD8^+$ T cells by *(i)* inhibiting their production of IL-2;*(ii)* repressing their expression of c-Myc, cyclin D2, and cyclin E; and *(iii)* stimulating the expression of the CDKIs p15, p21, and p27 [30, 39,40]. In contrast to its stimulation of cytostasis in $CD8^+$ T cells, TGF-β has no effect on the proliferation of $CD4^+$ T cells, but does inhibit the differentiation of $CD4^+$ T cells into Th1 and Th2 lineages by *(i)* downregulating T cell receptor expression; *(ii)* reducing intracellular Ca^{++} signaling; and *(iii)* repressing the expression and activation of transcription factors [30, 39,40], all of which weaken host immunosurveillance. Collectively, these findings predict that inactivating TGF-β signaling in $CD8^+$ or $CD4^+$ T cells will inhibit tumor formation by elevating host immunosurveillance, a supposition shown to occur during T cell-mediated eradication of skin [43] and prostate [44] cancers in mice. More recently, TGF-β was observed to promote the development and progression of breast and colon cancers by inducing CD8+ T cells to

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secrete IL-17, which exerts pro-survival signaling in carcinoma cells [45]. Thus, in addition to improving host immunosurveillance, neutralizing TGF-β function in T cells also will improve tumor resolution by suppressing the activation of carcinoma survival pathways.

1.2.2. TGF-β and Innate Immunity—In addition to its role in regulating adaptive immunity, TGF-β also plays an essential role in directing activities and behaviors of components of the innate immune system, including NK cells, dendritic cells, mast cells, monocytes, and macrophages. Indeed, we defined a novel TAB1:xIAP:TAK1:IKKβ:NF-κB signaling axis that forms aberrantly in breast cancer cells, and in normal MECs following their induction of EMT by TGF-β. Once formed, this signaling axis enables oncogenic signaling by TGF-β in part *via* activation of NF-κB and its consequential production of proinflammatory cytokines, which promote breast cancer growth in mice in a manner consistent with regulation of innate immunity by TGF-β [46]. Along these lines, TGF-β receptors were observed to associate with those for IL-1β, thereby enabling *(i)* TGF-β to activate NF-κB; *(ii)* IL-1β to activate Smad2; and *(iii)* both pathways to potentiate inflammatory cytokine production [47] their ability to promote inflammation and the enhanced the survival of tumor-associated monocytes [48,49]. In addition, transgenic expression of IL-1β in the stomachs of mice promoted the activation of myeloid-derived suppressor cells (MDSCs) *via* an IL-1R/NF-κB signaling axis, whose inappropriate and constitutive activation results in the formation of stomach neoplasias [50]. TGF-β is a potent inhibitor of the cytolytic activity of NK cells, presumably by attenuating the activation of their NKp30 and NKD2D receptors, and by inhibiting their production of interferon-γ. In addition, TGF-β also represses the activities of dendritic cells by inhibiting their expression of MHC class II, CD40, CD80, and CD86, and TNF-α, IL-12, and CCL5/Rantes [30,39,40,51]. Mast cells are actively recruited to tumor microenvironments by TGF-β where they synthesize and secrete numerous tumor promoting factors, including histamine, proteases, and cytokines (*e.g.*, VEGF and TGFβ) [40,52]. Lastly, TGF-β stimulates monocytes and macrophage chemotaxis to tumor microenvironments, leading to enhanced tumor invasion, angiogenesis, and metastasis, and to diminished antigen presentation and immunosurveillance towards developing neoplasms [53,54].

1.3. TGF-β and Endothelial Cells

Angiogenesis is the process whereby new blood vessels sprout and form from preexisting vessels; it also is an essential physiological process that transpires during embryonic development, wound healing, and the female reproductive cycle [55,56]. The initiation of pathological angiogenesis has been linked to numerous human diseases, including rheumatoid arthritis, diabetic retinopathy, and age-related macular degeneration [56,57]. Interestingly, all solid tumors larger than 1 cm^3 suffer from hypoxia [58], and as such, initiate angiogenesis as a means of acquiring an efficient supply of nutrients and waste removal, as well as a route for their metastasis to distant locales. Two distinct phases are involved in angiogenesis, namely angiogenic activation and resolution. During the activation phase of angiogenesis, ECs initially exhibit increased vessel permeability and elevated rates of cell proliferation, migration, and invasion. In addition, new vessel sprouting is further enhanced by a reduction in EC adhesion, coupled to an alteration in basement membrane integrity. In contrast, angiogenic resolution essentially restores activated ECs to their resting, quiescent phenotypes, as well as promotes the recruitment of perivascular cells that maintain vessel stability and hemodynamics [55–57].

TGF-β plays critical roles in regulating both the activation and resolution phases of angiogenesis [59–62]. Indeed, homozygous deletion of various components of the TGF-β signaling system in mice routinely results in the appearance of vascular and EC defects, particularly in animals lacking TGF-β1 [63], TβR-I [64], TβR-II [65,66], TβR-III [67,68], Smad1 [69], or Smad5 [70]. In humans, loss or inactivation of endoglin leads to hereditary

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hemorrhagic telangiectasia type 1 (HHT1) [71,72], while that of ALK1 results in HHT2 [73, 74]. Moreover, the defects associated with HHT1 and HHT2 in humans are phenocopied in knockout mice lacking expression of either endoglin [75,76] or ALK1 [77–79], respectively. Thus, altered expression and/or activity of TGF-β in tumor microenvironments clearly will impact the ability of hypoxic tumors overcome this impediment to their growth and survival.

ECs have been reported to express two distinct TβR-Is, namely TβR-I/Alk5 and ALK1. The importance of these two receptors in mediating vessel development by TGFβ is evidenced by the embryonic lethality observed at day E11.5 and E10.5 in mice lacking ALK1 [79] or ALK5 [64], respectively. Recent evidence also suggests that these two type I receptors differentially regulate the coupling of TGF-β to angiogenic activation and resolution. For instance, TβRI/ALK5 activation stimulates Smad2/3 and the subsequent expression of genes operant in mediating vessel maturation, including plasminogen activator inhibitor 1 (PAI-1) and fibronectin [78,80,81]. Moreover, microarray gene expression analyses of EC cells before and after their stimulation with TGF-β confirmed that the activation of a TGF-β:TβR-I/ALK5:Smad2/3 signaling axis does indeed promote angiogenic resolution [61,82]. In contrast, ALK1 activation stimulates Smad1/5 and the subsequent expression of genes operant in mediating angiogenesis activation, including Id1 and interleukin 1 receptor-like 1 [78,80–82]. Moreover, ALK-1 signaling stimulated by TGFβ requires this cytokine to initially activate TβR-II and ALK-5, which then recruit and activate ALK-1 following its association with TβR-II:ALK-5:TGF-β ternary complexes [78]. Thus, activation of ALK-1 and the induction of angiogenesis by TGF-β must first proceed through its assembly of angiostatic TGF-β receptor complexes (*i.e.*, TβR-II:ALK-5). At present, the molecular mechanisms that initially exclude and then recruit ALK-1 to angiostatic TGF-β receptor complexes remain unknown, but may reflect a delicate balance between TGF-β and other angiogenic factors located within tumor microenvironments. Indeed, low TGFβ concentrations enhance the ability of bFGF and VEGF to stimulate EC proliferation and angiogenic sprouting, while high TGF-β concentrations inhibit these angiogenic activities [62,83]. Along these lines, the pro-angiogenic functions of TGF-β also have been linked to its ability to regulate the expression and/or activities of other angiogenic factors, such as bFGF and VEGF [84]. It is interesting to note that inclusion of TGF-β to Matrigel plugs implanted into mice only promotes angiogenesis and vessel development in the presence of bFGF and its ability to create a pro-angiogenic microenvironment (M. Tian and W.P. Schiemann, *unpublished observation*). Thus, it is plausible that the recruitment of ALK-1 to angiostatic TGF-β receptor complexes may first require the stimulation of accessory angiogenic signals or proteins within activated EC microenvironments. Along these lines, the coupling of TGFβ to angiogenesis is controlled by the presence of endoglin, whose expression is induced by ALK1 and serves to promote EC proliferation, migration, and tubulogenesis by antagonizing the activities of TβR-I/ALK5 [60,82].

Collectively, these studies highlight the complexities associated with the ability of TGF-β to regulate EC activities coupled to angiogenesis. Future studies clearly need to *(i)* better define the precise mechanisms that enable TGFβ and its downstream effectors to govern the induction of angiogenic or angiostatic gene expression profiles; *(ii)* establish the impact of EC and perivascular cell differentiation states to influence the angiogenic response to TGF-β; and *(iii)* identify the microenvironmental cues and signals the cooperate with TGF-β in mediating angiogenesis activation and resolution.

2. TGF-β, EMT, and Metastasis

The acquisition of invasive and metastatic phenotypes by carcinomas ushers in their transition from indolent to aggressive disease states, during which time immotile, polarized epithelial cells undergo EMT and transdifferentiate into highly motile, apolar fibroblastoid-like cells

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[85–87]. In doing so, post-EMT carcinoma cells remodel their ECM and microenvironments in a manner that facilitates their intravasation into the vascular or lymphatic systems, as well as their extravasation at distant locales to form micrometastases that ultimately develop into secondary carcinomas [88]. Interestingly, a recent study identified a set of potential metastatic gene signature whose expression is highly associated with the acquisition of pulmonary metastasis by human breast cancers [89]. Included in this metastatic gene signatures are IDs (**I**nhibitor of **D**ifferentiation) 1 and 3, which mediate constitutive proliferative signals in newly established pulmonary micrometastases [89]. In addition, the ability of TGF-β to induce ANGPTL4 (angiopoietin-like 4) expression in breast cancer cells enables their retention, extravasation, and colonization specifically in the lungs, not the bone [90]. Pathological reactivation of EMT programs in differentiated cells and tissues not only promotes their invasion and metastasis, but also underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis, and chronic fibrotic degenerative disorders, all of which are characterized by dysregulated microenvironmental signaling [85–88,91,92]. In the following sections, we summarize recent developments linking TGF-β to the induction of EMT and metastasis, to the selection and expansion of cancer stem cells, and to the regulation of microRNA expression in developing and progressing neoplasms.

2.1. TGF-β Signaling and EMT

2.1.1. Canonical TGF-β Effectors and EMT—The ability of TGF-β to induce EMT and metastasis transpires through the activation of canonical (*i.e*., Smad2/3-dependent) and noncanonical (*i.e.,* Smad2/3-independent) TGF-β signaling inputs. For instance, inactivating canonical TGF-β signaling in human MCF10ACA1a breast cancer cells by engineering their expression of a dominant-negative Smad3 construct [93] or a TβR-I mutant incapable of activating Smad2/3 (*i.e*., L45 mutant) [94] significantly reduced their ability to colonize the lung. Along these lines, Smad4-deficiency not only diminished the expression of PTHrP, IL-11, and CTGF in human MDA-MB-231 breast cancer cells, but also abrogated their metastasis to bone in response to TGF-β [95–98]. Interestingly, whereas Smad4-deficiency cooperates with oncogenic K-Ras to induce the initiation and development of pancreatic cancer, the expression and activity of Smad4 is essential for TGFβ stimulation of pancreatic cancer EMT and growth [99]. Similar inactivation of canonical TGF-β signaling by overexpression of Smad7 [100, 101] prevents the invasion of breast [102] and head and neck cancers [103,104], as well as the pulmonary metastasis of melanomas [105]. Collectively, these findings highlight the importance of Smad2/3/4 signaling in mediating EMT and metastasis stimulated by TGF-β, and suggest the potential benefit of Smad2/3 antagonists to improve the clinical course of patients with metastatic disease.

2.1.2. Noncanonical TGF-β Effectors and EMT—Noncanonical TGF-β signaling also plays an essential role in mediating TGF-β stimulation of EMT, invasion, and metastasis [106]. Included in this growing list of noncanonical effectors targeted by TGFβ are Ras/MAP kinase [107–115], PI3K/AKT [116], Rho/ROCK [117], Jagged/Notch [118], mTOR [119], and Wnt/β-catenin [120]. Collaborative signaling events occurring between NFκB and oncogenic Ras also mediate EMT and pulmonary extravasation of breast cancer cells in response to TGF-β [121]. Similarly, we identified TGF-β stimulation of NF-κB as an essential pathway operant in coupling TGF-β to the expression of Cox-2, whose activity and production of PGE2 are critical for EMT induced by TGF-β in normal and malignant MECs [122]. We [23–25] and others [108] also established integrins as key players in mediating EMT, invasion, and p38 MAPK activation by TGF-β, as well as its ability to stimulate the growth and pulmonary metastasis of breast cancers in mice [25]. Essential effectors targeted by the formation of integrin:TGF-β receptor signaling complexes are *(i)* the protein proto-oncogene Src and its phosphorylation of TβR-II at Y284, which creates a docking site for Grb2 and ShcA [23–25]; *(ii)* the adapter molecule Dab2, which facilitates TGF-β stimulation of Smad2/3 and FAK

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[123,124]; and *(iii)* the protein tyrosine kinase FAK, which coordinates the formation of αvβ3 integrin:TβR-II complexes and, together with its effector p130Cas, is essential for TGF-β stimulation of breast cancer pulmonary metastasis in mice (M.K. Wendt and W.P. Schiemann, *unpublished observation*). In addition, αvβ3 integrin also mediates TGF-β-dependent metastasis of breast cancer cells to bone [125,126]. Collectively, these findings implicate TβR-II as an essential mediator of oncogenic signaling by TGF-β, particularly its ability to promote the acquisition of invasive and metastatic phenotypes at the expense of significantly impacting primary tumor growth [127]. Along these lines, a missense mutation in TβR-II identified in human head and neck carcinomas was observed to promote their EMT and invasion in part *via (i)* hyperactive protein kinase activity in mutant TβR-II proteins, and *(ii)* inappropriate coupling of TGF-β receptors to Smad1/5 activation, as opposed to Smad2/3 [128]. Interestingly, following its phosphorylation by TβR-II, the tight-junction assembly protein, PAR-6, associates with TβR-I and coordinates the ubiquitination and degradation of RhoA by Smurf1 [129]. The net effect of these TGF-β-dependent events results in the dissolution of epithelial cell tight junctions and the disassembly of their actin cytoskeleton, leading to the induction of EMT.

2.2. TGF-β and Cancer Stem Cells

It is important to note that EMT is a normal and essential physiological process that directs tissue development and morphogenesis in the embryo, as well as promotes the healing, remodeling, and repair of injured tissues in adults [85–87]. Thus, tumorigenic EMT in many respects reflects the inappropriate reactivation of embryonic and morphologic gene expression programs, and as such, points towards a potential link between EMT and the maintenance of stem cell properties. Accordingly, aggressive and poorly differentiated breast cancer and glioma cells exhibit gene signatures characteristic of stem cells [30], while human and mouse MECs induced to undergo EMT acquire stem cell-like properties in part *via* activation of the TGF-β signaling system [131]. Because TGF-β is a master regulator of physiological and pathological EMT [91], these findings suggest that the conversion of TGF-β from a tumor suppressor to a tumor promoter mirrors its ability to induce the selection and expansion of stem cell-like progenitors in post-EMT cells. In fact, TGF-β treatment of malignant, but nonmetastatic human breast cancer cells suppressed their tumorigenicity by diminishing the size of the cancer stem cell pool, and by reducing ID1 expression that results in the differentiation of the progenitor pool [132]. Thus, uncoupling TGF-β from regulation of ID1 expression may dictate whether TGF-β either promotes or suppresses the maintenance and/or expansion of cancer stem cells. Indeed, pharmacological inhibition of TGF-β signaling in cancer stem cells induced a mesenchymal-epithelial transition that resulted in their acquisition of a more epithelial-like morphology [133]. Along these lines, Future studies clearly need to *(i)* identify the molecular mechanisms that link TGF-β and EMT to the generation of cancer stem cells, and *(ii)* establish the therapeutic impact of TGF-β in promoting chemoresistance *via* its stimulation of EMT and the expansion of cancer stem cells.

2.3. TGF-β and microRNAs

Finally, accumulating evidence now positions microRNAs as potentially important regulators of the "TGF-β Paradox." Indeed, expression of miR-21 in breast cancers predicts for elevated TGF-β1 expression and a poor clinical prognosis [134], while that in gliomas results in the suppression of multiple components of the TGF-β signaling system, including its ligands (*e.g.*, TGF-βs 1 and 3), its receptors (*e.g.*, TβR-II and TβR-III), and its effector molecules (*e.g.*, Smad3, Daxx, and PDCD4) [135,136]. Recently, TGF-β was shown to promote contractile phenotypes in vascular smooth muscle cells by stimulating the processing of primary miR-21 transcripts into their pre-miR-21 counterparts *via* the formation of Smad2/3:DROSHA complexes. In doing so, cellular levels of miR-21 accumulate rapidly, resulting in diminished expression of PDCD4 (**p**rogrammed **c**ell **d**eath **4**) and its inability to

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suppress contractile machinery expression in vascular smooth muscle cells [136]. Similar induction of miR-21 expression took place in Smad4-deficient carcinoma cells, suggesting that TGF-β-regulated miR processing also takes place in epithelial cells in a manner independent of Smad4 [136]. Moreover, miR-21 expression also functions to promote EMT stimulated by TGF-β [137], although the molecular mechanisms underlying this event remain to be determined definitively. In contrast to miR-21 and its role in promoting EMT by TGF-β, microRNA-200 family members and miR-205 function in maintaining epithelial cell polarity and, consequently, in suppressing EMT. Importantly, the ability of TGFβ to induce EMT first requires this cytokine to downregulate microRNA-200 family member and miR-205 expression, which promotes ZEB1 and ZEB2 expression and their initiation of EMT [138]. Thus, aberrant microRNA expression may play a significant role in determining whether epithelial cells sense and respond to the tumor suppressing functions of TGFβ, or rather to its oncogenic activities.

Conclusions and Future Perspectives

Despite considerable progress over the last decade in defining the molecular mechanisms that underlie the initiation and maintenance of the "TGF-β Paradox," science and medicine still lack the necessary knowledge and wherewithal to explain and, more importantly, to manipulate the physiopathological actions of TGFβ to improve the clinical course of human malignancies. While it is abundantly clear that TGF-β plays a major role, both directly and indirectly, in regulating the ability of cancer cells to acquire each of the 6 hallmarks necessary for their malignant progression [139], it remains unclear as to how these events conspire in regulating the response of developing and progressing neoplasms to TGFβ. For instance, defects in TGF-β function rarely effect primary tumor growth, but more commonly play a significant role in enabling cancer cells to acquire EMT and invasive/metastatic phenotypes. Thus, while it is easy to rationalize why tumors require TGF-β to provide them with a selective EMT and metastatic advantage, teleologically it remains troublesome to assume that these phenotypic changes induced by TGF-β are permanently ingrained in aggressive carcinoma cells. Indeed, cancer cells perpetually locked into a "vagabond" mentally is counterintuitive to the processes underlying metastasis development and the formation of secondary carcinomas at distant locales. Instead, it appears that the exquisite balance between the functions and behaviors of TGF-β in distinct tissue types become unbalanced and incapable of suppressing disease development, particularly that of neoplastic transformation. Along these lines, the processes underlying the maintenance of normal tissue and cellular homeostasis have been liken to those necessary in facilitating the existence of a well-balanced and harmonious society [140]. The studies highlighted herein are consistent with a role for TGFβ in serving either as a benevolent or corrupt village manager, one whose ultimate agenda is dictated by the prevailing mood of the village's stromal and microenvironmental constituents. Thus, these findings also underscore and reinforce the need to develop novel pharmacological agents designed to antagonize the oncogenic activities of TGF-β in cancer cells, as well as in their supporting stromal compartments.

Executive Summary

TGF-β and the Tumor Microenvironment Fibroblasts

- Tumor reactive stroma plays a critical role in determining whether TGFβ suppresses or promotes tumor formation.
- Loss and/or disruption of paracrine signaling systems between fibroblast and adjacent epithelial cells results in cellular transformation, and in the progression of developing neoplasms.

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- Similar inactivation of TGF-β function in epithelial cells also elicits aberrant epithelial:fibroblast paracrine signaling networks that drives malignancy.
- TGF-β stimulation of desmoplastic and fibrotic reactions promotes the formation of stiff, noncompliant microenvironments that select for the expansion of metastatic cells.
- LOX family members are essential for desmoplasia induced by TGF-β, and for stimulating breast cancer metastasis in hypoxic tumor environments.
- Fibrotic reactions enhance TGF-β signaling and may facilitate tumor protection to radiotherapies.

TGF-β and Immunosurveillance

- TGF-β is a potent suppressor of inflammation and immune suppression.
- Similar to fibroblasts, altered paracrine signaling by immune cell contributes to tumor formation, particularly that in the gastrointestinal track.
- TGF-β is a potent inhibitor of adaptive immunity, which contributes to weaken host immunosurveillance.
- TGF-β also is a potent activator of innate immunity, which contributes to carcinoma progression and metastasis.

TGF-β and Endothelial Cells

- Angiogenesis is the process whereby new blood vessels develop from pre-existing vessels.
- Angiogenesis also provides cancer cells a route for their metastatic spread.
- Aberrant TGF-β signaling elicits developmental vascular defects that typically result in embryonic lethality.
- Human hereditary hemorrhagic telangiectasia (HTT) is phenocopied in mice lacking expression of either ALK1 or endoglin.
- TGF-β regulates both the activation and resolution of angiogenesis by differential activation of ALK1 (*i.e*., pro-angiogenic *via* Smad1/5/8 activation) or TβR-I/ALK5 (*i.e*., anti-angiogenic *via* Smad2/3 activation).
- Activation of ALK1 by TGF-β requires the presence of ALK5 and TβR-II.

TGF-β, EMT, and Metastasis

- EMT is a process whereby polarized, immotile epithelial cells transdifferentiate into apolar, highly motile fibroblastoid-like cells.
- TGF-β is a master regulator of normal and tumorigenic EMT.
- EMT is essential for the acquisition of invasive and metastatic phenotypes in carcinoma cells.
- TGF-β induces EMT *via* stimulation of canonical (*i.e*., Smad2/3) and noncanonical (*i.e*., Ras/MAP kinases, PI3K, AKT, and Rho/ROCK) pathways.
- β3 integrin, Src, and p38 MAPK are essential in facilitating EMT stimulated by TGF-β.

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- Aberrant expression of microRNAs in response to TGF-β may drive EMT and metastasis.
- EMT induced by TGFβ may play important roles in generating chemoresistant cancer stem cells.

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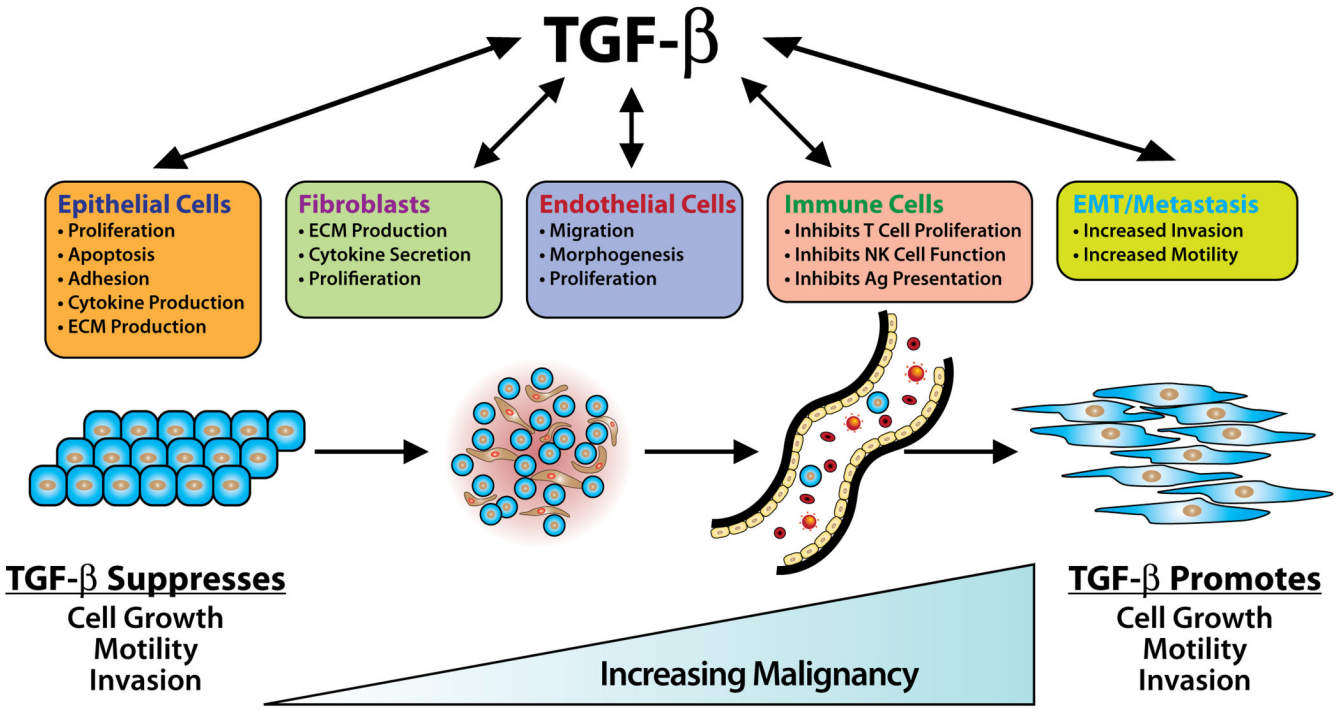

Figure 1. Cellular Targets of TGF-β During the Development and Progression of Human Cancers

TGF-β is a multifunctional cytokine that normally suppresses cell proliferation, differentiation, and apoptosis, as well as regulates cell and tissue homeostasis. Under normal physiological conditions, TGF-β functions as a tumor suppressor by preventing the ability of cells to progress through the cell cycle, or by stimulating the ability of cells to undergo apoptosis or differentiation. However, genetic and epigenetic events that transpire during tumorigenesis can convert TGF-β from a tumor suppressor to a tumor promoter, particularly the ability of cancer cells to acquire invasive and metastatic phenotypes. The oncogenic activities of TGF-β also are coordinated by dysregulated autocrine and paracrine signaling networks that take place between epithelial, fibroblasts, endothelial, and immune cells, that collectively promote tumor angiogenesis, invasion, and metastasis, and inhibit host immunosurveillance within tumor microenvironments. See text for specific examples of how TGF-β signaling becomes dysregulated during tumorigenesis

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p130Cas Is Required for Mammary Tumor Growth and Transforming Growth Factor-β-mediated Metastasis through Regulation of Smad2/3 Activity*[S]

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During breast cancer progression, transforming growth factor-β (TGF-β) switches from a tumor suppressor to a pro-metastatic molecule. Several recent studies suggest that this conversion in TGF-β function depends upon fundamental changes in the TGF-β signaling system. We show here that these changes in TGF-β signaling are concomitant with aberrant expression of the focal adhesion protein, p130Cas. Indeed, elevating expression of either the full-length (FL) or just the carboxyl terminus (CT) of p130Cas in mammary epithelial cells (MECs) diminished the ability of TGF-β1 to activate Smad2/3, but increased its coupling to p38 MAPK. This shift in TGF-β signaling evoked (i) resistance to TGF-β-induced growth arrest, and (ii) acinar filling upon three-dimensional organotypic cultures of p130Cas-FL or -CT expressing MECs. Furthermore, rendering metastatic MECs deficient in p130Cas enhanced TGF-β-stimulated Smad2/3 activity, which restored TGF-β-induced growth inhibition both *in vitro* and in mammary tumors produced in mice. Additionally, whereas elevating TβR-II expression in metastatic MECs had no affect on their phosphorylation of Smad2/3, this event markedly enhanced their activation of p38 MAPK, leading to increased MEC invasion and metastasis. Importantly, depleting p130Cas expression in TβR-II-expressing metastatic MECs significantly increased their activation of Smad2/3, which (i) reestablished the physiologic balance between canonical and noncanonical TGF-β signaling, and (ii) reversed cellular invasion and early mammary tumor cell dissemination stimulated by TGF-β. Collectively, our findings identify p130Cas as a molecular rheostat that regulates the delicate balance between canonical and noncanonical TGF-β signaling, a balance that is critical to maintaining the tumor suppressor function of TGF-β during breast cancer progression.

Invasion and metastasis are the most lethal characteristics of breast cancer (1, 2). Transforming growth factor-β (TGF-β)[2] is a powerful suppressor of mammary tumorigenesis, doing so through its ability to repress mammary epithelial cell (MEC) proliferation, as well as through its creation of cell microenvironments that inhibit MEC motility, invasion, and metastasis (2). During breast cancer progression, the tumor suppressing function of TGF-β is frequently subverted, thus transforming TGF-β from a suppressor of breast cancer formation to a promoter of its growth and metastasis (2–4). Unfortunately, how mammary tumorigenesis overcomes the cytostatic function of TGF-β remains incompletely understood, as does the manner in which developing breast cancers ultimately sense TGF-β as a pro-metastatic factor.

Transmembrane signaling by TGF-β commences upon binding to its type II receptor (TβR-II), which recruits and activates its type I receptor (TβR-I), which then phosphorylates and activates Smads 2 and 3. Following their activation, Smads 2 and 3 form heteromeric complexes with Smad4, which collectively translocate to the nucleus to regulate a multitude of transcriptional events and cellular responses (*i.e.* apoptosis, cytostasis, and homeostasis, (5, 6)). In addition to stimulating Smad2/3, TGF-β also activates several noncanonical signaling systems, including members of the MAP kinase family (*e.g.* ERK1/2, JNK, and p38 MAPK (7)). Interestingly, several studies suggest that genetic and epigenetic events cooperate with aberrant Smad2/3 activities and functions to facilitate the conversion of TGF-β from tumor suppressor to a tumor promoter (8, 9). However, these and other studies also present strong evidence implicating dysregulated activation of several noncanonical TGF-β effectors during this same switch in TGF-β function (10). Thus, deciphering the relative contribution of signaling imbalances that arise between Smad2/3-dependent and -independent TGF-β signaling systems is essential to enhancing our understanding of how TGF-β ultimately promotes the development and progression of mammary tumorigenesis.

Recently, we identified a critical αvβ3 integrin:pY284-TβR-II:Grb2 signaling axis that mediates TGF-β stimulation of MAP kinases in normal and malignant MECs, leading to their acquisition of epithelial-mesenchymal transition, invasive, and metastatic phenotypes both *in vitro* and *in vivo* (11–13). Moreover, activation of this oncogenic signaling axis by TGF-β requires

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[2] The abbreviations used are: TGF-β, transforming growth factor-β; FAK, focal adhesion kinase; MEC, mammary epithelial cell; TβR-I, TGF-β type I receptor; TβR-II, TGF-β type II receptor; p130Cas, Crk-associated substrate; PAI-1, plasminogen activator inhibitor-1; NmuMG, normal murine mammary epithelial; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; JNK, c-Jun N-terminal kinase; CMV, cytomegalovirus; shRNA, short hairpin RNA; β-gal, β-galactosidase; FL, full-length; CT, carboxyl-terminal.

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β3 integrin to form complexes with TβR-II (11–13). Unfortunately, it remains uncertain as to whether this interaction is direct or facilitated through another scaffolding protein. As such, we sought to identify members of focal adhesion complexes as potential integrin effectors capable of contributing to altered TGF-β signaling.

p130Cas (Crk-associated substrate) functions as a molecular scaffold within focal adhesion complexes, and is readily phosphorylated by focal adhesion kinase (FAK) and Src (14). Additionally, p130Cas binds stably to a variety of signaling molecules, including the (i) protein-tyrosine kinases FAK, PYK2, Src, Fyn, and Abl; (ii) adaptor molecules Crk, CrkL, Trip6, and AJUBA; (iii) guanine nucleotide exchange factors AND34 and CG3; and (iv) the MAPK family member, JNK (15, 16). The extensive interactome of p130Cas ideally positions and enables this molecule to interpret and integrate a variety of signaling inputs arising from numerous receptor systems. Indeed, the biological importance of p130Cas is emphasized by studies showing that its genetic ablation in mice elicits embryonic lethality, whereas fibroblasts derived from p130Cas-deficient embryos exhibit drastically altered cytoskeletal architectures (17). Moreover, fibroblasts transformed by Src become significantly more invasive when engineered to simultaneously overexpress p130Cas (15). Patients with primary breast tumors expressing high levels of p130Cas (also known as breast cancer resistance-1) experience a more rapid disease recurrence and have a greater risk of resistance to tamoxifen therapy (18). Recent studies also indicate that specific overexpression of p130Cas/breast cancer resistance-1 expression can confer breast cancer resistance to adriamycin (19). Moreover, directed overexpression of p130Case in murine MECs significantly increased their proliferative and survival indices *in vivo*, as well as greatly reduced the latency of mammary tumors arising from murine mammary tumor virus-driven Her2/Neu expression in mice (20). This study also observed the expression of p130Cas to be up-regulated significantly in a subset of human breast cancer samples (20). Collectively, these findings highlight the critical roles played by p130Cas in regulating normal tissue morphogenesis, and in promoting breast cancer progression. With respect to TGF-β, a recent study identified p130Cas as a potential inhibitor of Smad3 function (21). However, the pathophysiological importance of this event, if any, in mediating the oncogenic activities of TGF-β and/or p130Cas during breast cancer progression remains to be established.

The objective of the present study was to determine the role of p130Cas in facilitating the acquisition of oncogenic signaling by TGF-β during breast progression. We show here that p130Cas expression is up-regulated significantly in metastatic breast cancer cells (murine 4T1/human MCF10A-Ca1a) as compared with their nonmetastatic counterparts (murine 67NR/human MCF10A). Moreover, increased p130Cas expression was consistent with a decrease in TGF-β1-induced Smad2/3 signaling. Indeed, overexpression of p130Cas in nonmetastatic MECs led to a decrease in Smad2/3 activity, whereas depletion of p130Cas in metastatic MECs increased Smad2/3 activity. Most importantly, we show for the first time that p130Cas is essential for TGF-β stimulation of breast cancer growth, invasion, and pulmonary dissemination in mice. Taken together, our findings establish p130Cas as a novel molecular rheostat that regulates the balance between canonical and noncanonical TGF-β signaling in developing mammary tumors, whose acquisition of metastatic phenotypes is potentiated by elevated p130Cas expression and its consequential disruption of homeostatic TGF-β signaling.

EXPERIMENTAL PROCEDURES

Cell Lines and Reagents—Normal murine NMuMG and metastatic 4T1 cells were obtained from ATCC (Manassas, VA) and cultured as described previously (12). 4T1 cells were engineered to stably express firefly luciferase by transfection with pNifty-CMV-luciferase (22), followed by their selection and isolation with Zeocin (500 μg/ml; Invitrogen). The creation of 4T1 cells lacking p130Cas was accomplished by their transduction with lentiviral particles encoding either a scrambled (*i.e.* non-silencing shRNA) or murine-directed p130Cas shRNA (pLKO.1; Thermo Scientific, Huntsville, AL). The production of pLKO.1 lentiviral particles and their transduction into target cells was accomplished as described previously (23). In addition, NMuMG and 4T1 cells also were transduced with murine ecotropic retroviral particles that encoded for full-length p130Cas (pMSCV-Cas-FL), the carboxyl terminus of p130Cas (amino acids 544–874) (pMSCV-Cas-CT), or TβR-II (pMSCV-TβR-II), and the resulting polyclonal populations were selected by yellow fluorescence protein, or hygromycin resistance (200 μg/ml).

Cell Proliferation Assays—NMuMG and 4T1 cells were seeded in 96-well plates (10,000 cells/well) and allowed to adhere for 4 h, whereupon varying concentrations of TGF-β1 (0–5 ng/ml) were administered. Agonist stimulations were allowed to proceed for 48 h at 37 °C and cellular DNA was radiolabeled by inclusion of [^{3}H]thymidine (1 μCi/well) during the final 6 h of TGF-β1 treatment. Afterward, the amount of [^{3}H]thymidine incorporated into cellular DNA was quantified by scintillation counting.

Immunoblot Assays—NMuMG and 4T1 cells were lysed on ice in three-dimensional RIPA buffer (50 mM Tris, 150 mM NaCl, 0.25% (v/v) sodium deoxycholate, 0.1% SDS (v/v), pH 7.4) supplemented with (i) protease inhibitor mixture (Sigma), and (ii) the phosphatase inhibitors sodium orthovanadate (10 mM), β-glycerophosphate (40 mM), and sodium fluoride (20 mM). Afterward, the resulting whole cell extracts were clarified by microcentrifugation prior to being immunoblotted with the following primary antibodies (dilution): (*a*) anti-phospho-p38 MAPK (1:500; Cell Signaling, Danvers, MA); (*b*) anti-p38 MAPK (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); (*c*) anti-phospho-Smad2 (1:1000; Cell Signaling); (*d*) anti-phospho-Smad3 (1:500; Cell Signaling); (*e*) anti-Smad 2/3 (1:1000; BD Biosciences); (*f*) anti-FAK (1:1000; Santa Cruz Biotechnology); (*g*) anti-p130Cas (1:1000; BD Biosciences); (*h*) phospho-p130Cas (1:1000; Cell Signaling); (*i*) anti-actin (1:1000; Santa Cruz Biotechnology); (*j*) lamin A/C (1:1000; Santa Cruz Biotechnology); and (*k*) E-Cadherin (1:2000; BD Biosciences).

Real-time PCR Analyses—Quiescent 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the p38 MAPK inhibitor SB208530 (10 μM) and total RNA was isolated using the RNeasy Plus Kit (Qiagen, Valen-

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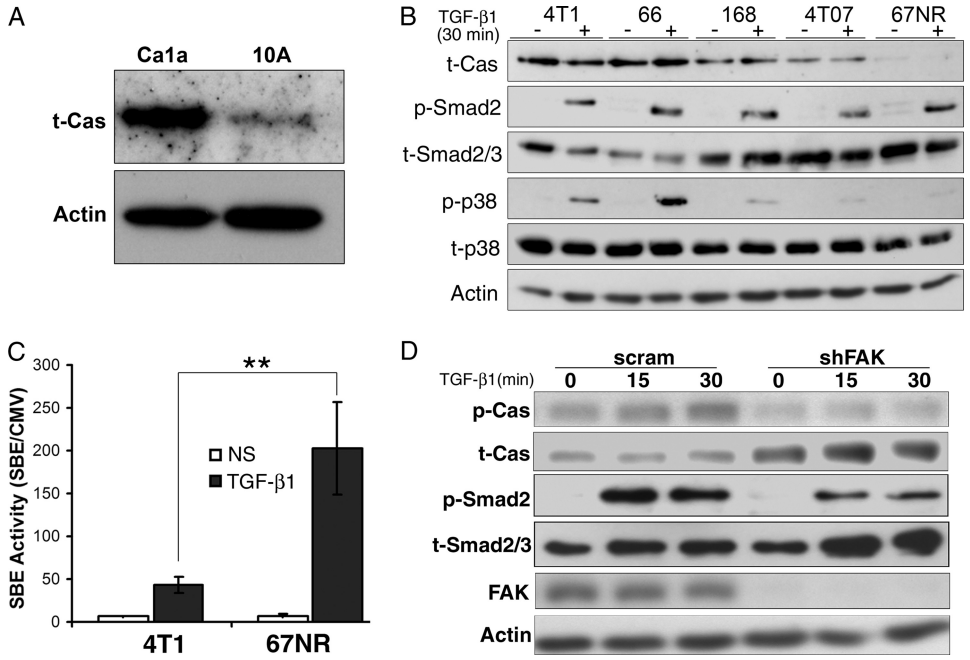

FIGURE 1. **Elevated p130Cas inhibits TGF-β-mediated Smad2/3 activation.** *A*, normal human MECs (*MCF-10A*) and their metastatic derivatives (*Ca1a*) were immunoblotted for p130Cas expression. β-Actin (*Actin*) is shown as loading control. Data are representative images from a representative experiment that was performed two times with identical results. *B*, murine breast cancer cells derived from the same primary Balb/c tumor, including the highly metastatic 4T1 and 66c14 (*66*) cells, the partially metastatic 168Farn (*168*) and 4T07 cells, and the nonmetastatic 67NR cells were immunoblotted for p130Cas expression, and the phosphorylation of Smad2 (*p-Smad2*) and p38 MAPK (*p-p38*) in response to TGF-β1 stimulation (5 ng/ml). Total Smad2/3 (*t-Smad2/3*), p38 MAPK (*t-p38*), and β-actin (*Actin*) were analyzed as loading controls. Data are from a representative experiment that was performed two times with identical results. *C*, 4T1 and 67NR cells were transiently transfected with pSBE-luciferase and pCMV-β-gal plasmids, and subsequently stimulated with TGF-β1 (5 ng/ml) for 18 h prior to measuring luciferase and β-gal activities. *NS*, no stimulation. Data are the mean ± S.E. ($n = 3$) of luciferase/β-gal activity ratios. **, $p = 0.01$. *D*, quiescent 4T1 cells that expressed either a scrambled (*scram*) or FAK-specific (*shFAK*) shRNA were stimulated with TGF-β1 (5 ng/ml) for varying times, and subsequently immunoblotted with phospho-specific antibodies against p130Cas (*p-Cas*) and Smad2 (*p-Smad2*) as indicated. Membranes were stripped and reprobed with antibodies against p130Cas (*t-Cas*), Smad2/3 (*t-Smad2/3*), β-actin (*Actin*), and FAK as indicated. Data are from a representative experiment that was performed at least three times with similar results.

cia, CA). Afterward, total RNA was reverse transcribed using the iScript cDNA Synthesis System (Bio-Rad) and semi-quantitative real-time PCR was conducted for PAI-1 using iQ SYBR Green (Bio-Rad) according to the manufacturer's recommendations and as described previously (23). Differences in RNA concentrations were controlled by normalizing individual gene signals to their corresponding glyceraldehyde-3-phosphate dehydrogenase signal.

Cell Fractionation Studies—Unstimulated and TGF-β1 (5 ng/ml)-stimulated NMuMG cells were lysed on ice in Buffer C (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 0.004% Nonidet P-40, pH 7.9) supplemented with protease inhibitor mixture (Sigma). Afterward, the resulting whole cell extract was subjected to a single freeze-thaw cycle, followed by microcentrifugation to yield a clarified cytoplasmic fraction. The remaining pellet was resuspended in Buffer N (20 mM HEPES, 400 mM NaCl, 1 mM EDTA, and 10% glycerol, pH 7.9) supplemented with protease inhibitor mixture, and shaken vigorously for 2 h at 4 °C. Afterward, this mixture was subjected to microcentrifugation to yield a clarified nuclear fraction.

Three-dimensional Culture Assays—NMuMG (1×10^4) and 4T1 (5×10^3) cells were diluted in complete medium supplemented with 5% Cultrex (R&D Systems, Minneapolis, MN), and subsequently seeded in 48-well plates on top of a Cultrex cushion. Where indicated, 4T1 cells were grown in the presence of TGF-β1 (5 ng/ml). The medium/Cultrex mixture was replaced at 7 days, and organoids were allowed to grow for a total length of 10 days, at which point they were monitored for hollowing by phase-contrast microscopy and quantified by three individuals who were blinded to the culture conditions. 4T1 acinar size was quantified using Image J software.

Cell Invasion Assays—The ability of TGF-β1 (5 ng/ml) to alter the invasion of 4T1 cells (50,000 cells/well) was analyzed using a modified Boyden Chamber assay as described previously (13).

Luciferase Reporter Gene Assays—NMuMG cells were transiently transfected overnight with LT1 liposomes (Mirus, Madison, WI) that contained 300 ng/well of pSBE-firefly luciferase (4X-CAGA) cDNA and 50 ng/well of pCMV-β-gal cDNA. Afterward, the cells were stimulated for 24 h with TGF-β1 (5 ng/ml), and subsequently harvested and assayed for firefly luciferase (Promega, Madison WI) and β-gal (Clontech, Mountain View, CA) activities. In addition, 4T1 cells that stably expressed firefly luciferase under control of the CMV promoter were similarly (i) transfected with 300 ng/well of pSBE-*Renilla* luciferase (4X-CAGA); (ii) stimulated with TGF-β1; and (iii) assayed for *Renilla* and firefly luciferase using the Dual-Glo Assay System as above (Promega).

Immunofluorescent Analyses—4T1 cells (25,000 cells/well) were allowed to adhere overnight to glass coverslips. Afterward, the cells were washed extensively in phosphate-buffered saline and immediately stimulated with TGF-β1 (5 ng/ml). Upon completion of agonist stimulation, the cells were (i) fixed in 4% paraformaldehyde; (ii) permeablized in 0.1% Triton X-100; (iii) stained with anti-Smad 2/3 antibodies (1:100; BD Biosciences); and (iv) visualized by addition of biotinylated anti-mouse antibodies (1:1000) in conjunction with the addition of rhodamine-conjugated streptavidin (1:2000).

Tumor Growth, in Vivo Bioluminescent Imaging, and Immunohistochemical Analyses—Control or various 4T1 derivatives engineered to stably express firefly luciferase were resuspended in sterile phosphate-buffered saline (50 μl) and injected orthotopically into the mammary fatpad (10,000 cells/injection) of 6-week-old female Balb/c mice (Jackson Laboratory, Bar Harbor, ME). Primary 4T1 tumor growth and metastasis development were assessed by (i) weekly bioluminescent imaging of

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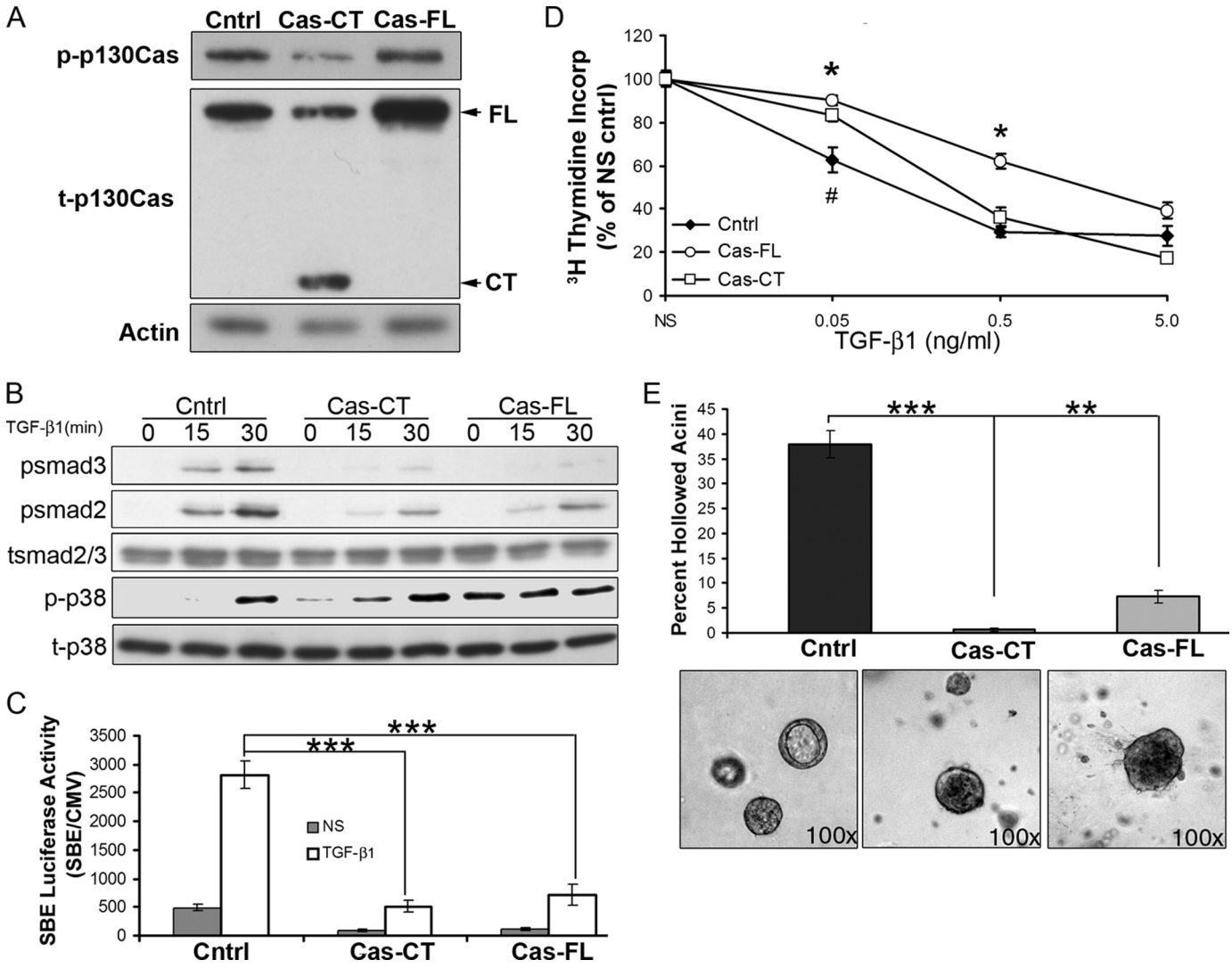

FIGURE 2. **Overexpression of p130Cas inhibits Smad2/3 activity and alters normal mammary epithelial acinar formation.** *A,* NMuMG cells were transduced with retroviral particles containing vector control (*cntrl*), full-length p130Cas (*Cas-FL*), or the carboxyl terminus of p130Cas (*Cas-CT*). Stable transgene expression was assessed by immunoblotting for phosphorylated (*p-p130Cas*) and total p130Cas (*t-p130Cas*). β-Actin (*Actin*) is shown as a loading control. *B*, the p130Cas-manipulated cell lines as described in *A* were stimulated with TGF-β1 (5 ng/ml) for the indicated times, and subsequently analyzed for the phosphorylation of Smad2 (*psmad2*), Smad3 (*psmad3*), and p38 MAPK (*p-p38*). Membranes were stripped and reprobed for total Smad2/3 (*tsmad2/3*) and p38 MAPK (*t-p38*) as loading controls. Data are from representative experiments that were performed at least three times with identical results. *C,* control (cntrl) and p130Cas-expressing (Cas-CT and Cas-FL) NMuMG cells were transiently co-transfected with pSBE-luciferase and pCMV-β-gal plasmids, and subsequently stimulated overnight with TGF-β1 (5 ng/ml) prior to measuring luciferase and β-gal activities. *NS*, no stimulation. Data are the mean ± S.E. of SBE/CMV activity ratios observed in three independent experiments completed in triplicate. ***, $p < 0.001$). *D*, control (*cntrl*) and p130Cas-expressing (*Cas-CT* and *Cas-FL*) NMuMG cells were stimulated with increasing concentrations of TGF-β1 (0–5 ng/ml) for 48 h, and subsequently assayed for [^{3}H]thymidine incorporation into cellular DNA. Data are the mean ± S.E. quantities of incorporated [^{3}H]thymidine normalized to unstimulated controls observed in three independent experiments completed in triplicate (*, $p < 0.05$ between Cntrl and Cas-FL; #, $p < 0.05$ between Cntrl and Cas-CT). *E*, the p130Cas-manipulated NMuMG cells described in *A* were grown in three-dimensional organotypic cultures for 10 days, at which point the percentage of hollowed acini were quantified by phase-contrast microscopy (**, $p < 0.001$; ***, $p < 0.0001$). Representative acini are shown.

tumor bearing animals on a Xenogen IVIS-200 (Xenogen Corporation, Hopkinton, MA); (ii) calculating primary tumor volumes using digital calipers and the equation digital V = $(x^2)(y)(0.5)$, where x is the tumor width and y is tumor length; and (iii) measuring primary tumor weights following their surgical excision on days 21 or 26 post-inoculation. Finally, serial histological sections of control and p130Cas-deficient 4T1 tumors were stained with Ki67 antibodies, and counterstained with hematoxylin as described previously (11). Data were quantified using Image J software. All animal studies were performed in accordance with the animal protocol procedures approved by the Institutional Animal Care and Use Committee of University of Colorado.

Statistical Analysis—Statistical values were defined using an unpaired Student's *t* test, where a p value < 0.05 was considered significant.

RESULTS

Elevated p130Cas Expression Inhibits TGF-β-mediated Smad2/3 Activation—Elevated expression of p130Cas is associated with mammary tumor progression (20), and with the uncoupling of TGF-β to Smad3 activation in epithelial cells

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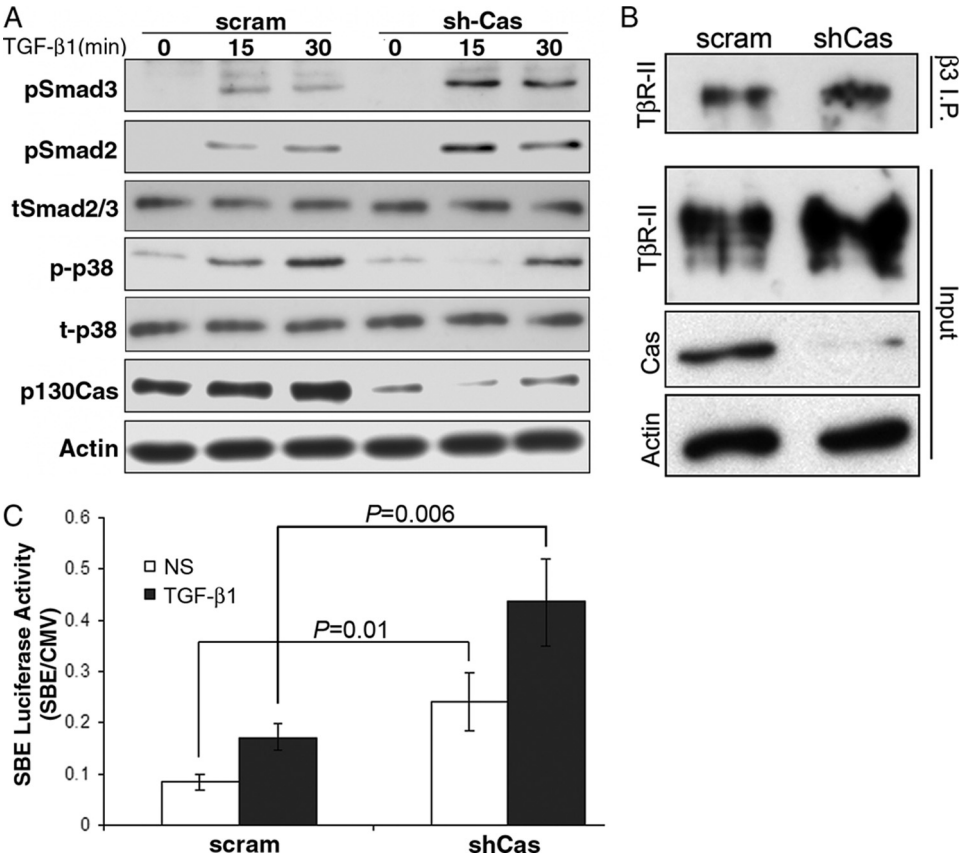

FIGURE 3. **p130Cas deficiency increases Smad2/3 activity in metastatic MECs.** *A*, quiescent 4T1 cells that expressed either a scrambled (*scram*) or p130Cas-specific (*shCas*) shRNA were stimulated with TGF-β1 (5 ng/ml) as indicated, and subsequently immunoblotted with phosphospecific antibodies against Smad3 (*pSmad3*), Smad2 (*pSmad2*), or p38 MAPK (*p-p38*) as shown. Membranes were stripped and reprobed with antibodies against Smad2/3 (*tSmad2/3*), p38 MAPK (*t-p38*), β-actin (*Actin*), and p130Cas as loading controls. *B*, whole cell extracts prepared from control (*scram*) or p130Cas-deficient (*shCas*) 4T1 cells were incubated with β3 integrin antibodies (*β3 I.P.*), and the resulting immunocomplexes were isolated and immunoblotted for TβR-II. Immunoblotting aliquots of the prepared cell extracts (*Input*) served to monitor the levels of TβR-II, p130Cas, and β-actin (*Actin*). Data are representative of three independent experiments and show that p130Cas deficiency does not affect carcinoma-specific formation of β3 integrin·TβR-II complexes. *C*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were transiently transfected with SBE-luciferase (*Renilla*), and subsequently stimulated overnight with TGF-β1 (5 ng/ml). *NS*, no stimulation. Data are the mean ± S.E. ($n = 3$) of *Renilla*/firefly activity ratios.

(21). Unfortunately, the pathophysiological importance of these events, if any, in mediating oncogenic TGF-β signaling in normal and malignant MECs remains unknown. As an initial measure of potential changes in p130Cas expression during mammary tumor progression, we monitored p130Cas protein levels by immunoblotting whole cell extracts prepared from nontransformed human MECs (MCF10A) and their corresponding metastatic derivatives (CA1a) (24–26). As shown in Fig. 1*A*, p130Cas expression was readily increased in human metastatic CAla cells as compared with nontransformed isogenic counterparts. Along these lines, we also observed p130Cas expression to be up-regulated dramatically in the murine 4T1 model of mammary tumor progression (Fig. 1*B*) (26, 27). Indeed, the highly metastatic 4T1 and 66c14 cells expressed significantly more p130Cas than did their moderately metastatic counterparts, 168-Farn and 4T07 (Fig. 1*B*). Consistent with this trend, we found nonmetastatic 67NR cells to express very little p130Cas (Fig. 1*B*). Importantly, the increased expression of p130Cas was consistent with a shift in the balance of TGF-β signaling from primarily that of canonical Smad2 phosphorylation in nonmetastatic 67NR cells to one that included a marked activation of p38 MAPK in metastatic 4T1 and 66c14 cells (Fig. 1*B*). Furthermore, using a measure of the downstream transcriptional activity of Smad2/3, we also observed a drastic diminution in the ability of TGF-β1 to activate Smad2/3 in 4T1 *versus* 67NR cells (Fig. 1*C*). We also monitored changes in the phosphorylation status of p130Cas upon stimulation with TGF-β. As shown in Fig. 1*D*, stimulating 4T1 cells with TGF-β not only readily induced the phosphorylation of Smad2, but also that of p130Cas (Fig. 1*D*). Moreover, the basal levels as well as the ability of TGF-β to induce phosphorylation of p130Cas were abrogated by rendering 4T1 cells deficient in its upstream kinase, FAK (Fig. 1*D*). Interestingly, depletion of FAK elicited a compensatory up-regulation of total p130Cas expression that was consistent with diminished coupling of TGF-β to Smad2 phosphorylation (Fig. 1*D*). Together, these findings are consistent with the notion that p130Cas, irrespective of its phosphorylation status, functions to shift the balance of TGF-β signaling during breast cancer progression by suppressing Smad2/3 activity and supporting p38 MAPK activation in response to TGF-β.

Overexpression of p130Cas Inhibits Smad2/3 Activity and Alters Normal Mammary Epithelial Acinar Formation—To address the role p130Cas in regulating TGF-β signaling, we overexpressed either the full-length protein (Cas-FL) or just the carboxyl terminus of p130Cas (Cas-CT) in normal murine mammary epithelial (NMuMG) cells (Fig. 2*A*). Indeed, overexpression of either Cas-CT or Cas-FL readily decreased the phosphorylation of Smad2 and Smad3 in response to TGF-β1 (Fig. 2*B*). In contrast, TGF-β-induced p38 MAPK phosphorylation was readily increased upon Cas-CT expression, whereas expression of Cas-FL was sufficient to induce the phosphorylation of p38 MAPK even in the absence of added TGF-β (Fig. 2*B*). Furthermore, overexpression of either Cas-CT or Cas-FL also dramatically decreased the extent of basal and TGF-β-induced Smad2/3-dependent transcription (Fig. 2*C*). Functionally, we observed the p130Cas-dependent reduction in Smad2/3 activity to significantly inhibit the cytostatic response of NMuMG cells to TGF-β (Fig. 2*D*). Finally, because TGF-β is critically involved regulating normal mammary gland develop-

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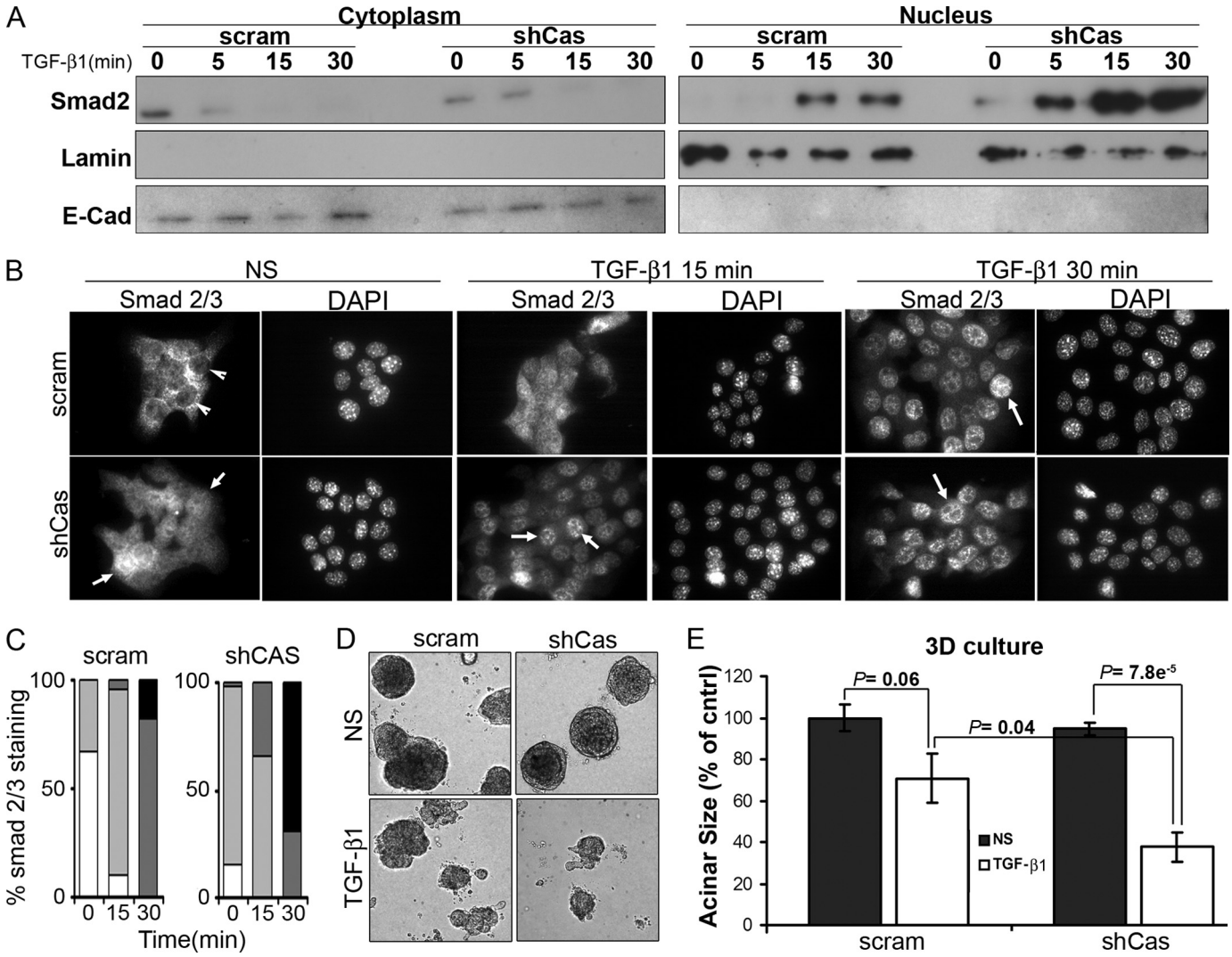

FIGURE 4. **p130Cas deficiency increases Smad2/3 nuclear localization and decreases the proliferation of metastatic MECs.** *A*, quiescent control (*scram*) or p130Cas-deficient (*shCas*) 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for the indicated times, and subsequently lysed, fractionated, and immunoblotted for Smad2/3. Membranes were stripped and reprobed with antibodies against E-cadherin (*E-Cad*) and lamin A/C (*Lamin*) to monitor the integrity of the cytoplasmic and nuclear preparations, respectively. Data are from a representative experiment that was performed three times with similar results. *B*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were stimulated as described in *A* and subsequently fixed and processed for indirect Smad2/3 immunofluorescence, as well as counterstained with 4′,6-diamidino-2-phenylindole (*DAPI*) to visualize cell nuclei. *Arrows* indicate the distinct absence or presence of Smad2/3 in 4T1 cell nuclei. Data are representative images from four independent experiments. *C*, Smad2/3 immunofluorescence data in *B* was quantified as follows: *white bars*, nuclear exclusion; *light gray bars*, cellular diffuse; *dark gray bars*, weak nuclear; *black bars*, strong nuclear staining for Smad2/3. Data are from 10 randomly selected fields for each time point obtained in two independent experiments. *D*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were grown in a three-dimensional organotypic culture for 10 days in the absence or presence of TGF-β1 (5 ng/ml). Representative acini from three independent experiments are shown. *E*, p130Cas depletion restored the cytostatic response of TGF-β in 4T1 cells, which were grown as in *D* prior to quantifying acinar size. Data are the mean ± S.E. of 9 randomly selected fields obtained from three independent experiments.

ment (28–30), we next sought to assess the affect of p130Cas overexpression on the formation of acini by NMuMG cells propagated in a three-dimensional organotypic culture system. Importantly, expression of either Cas-FL or Cas-CT readily invoked acinar filling, a phenotype that recapitulates *in vivo* mammary tumor progression (31). Taken together, these findings clearly indicate that inappropriate up-regulation (see Fig. 1) of p130Cas expression was sufficient to inhibit the physiologic activity of Smad2/3, thereby diminishing the tumor suppressive activities of TGF-β.

p130Cas Deficiency Increases Smad2/3 Activity in Metastatic MECs—Given that elevating p130Cas expression was sufficient to inhibit Smad2/3 signaling stimulated by TGF-β, we next examined how p130Cas deficiency would affect the coupling of Smad2/3 to TGF-β in MECs. To this end, we expressed and screened five independent p130Cas-specific shRNA sequences in NMuMG cells. The general importance of p130Cas in maintaining normal MEC physiology and homeostasis was readily apparent as NMuMG cells that expressed shCas#5 shRNA, which elicited the greatest degree of p130Cas depletion (see supplemental Fig. S1*A*), failed to thrive and survive under extended culture conditions (data not shown). Overall, p130Cas deficiency led to decreased Smad2 expression in NMuMG cells (see supplemental Fig. S1*A*). These findings are consistent with the notion that p130Cas deficiency augments the activity of Smad2/3, which elicits proteosome-directed degradation of Smad2/3 (see supplemental Fig. S1*B*) (23, 32, 33). However, this decrease in Smad2 expression precluded a direct

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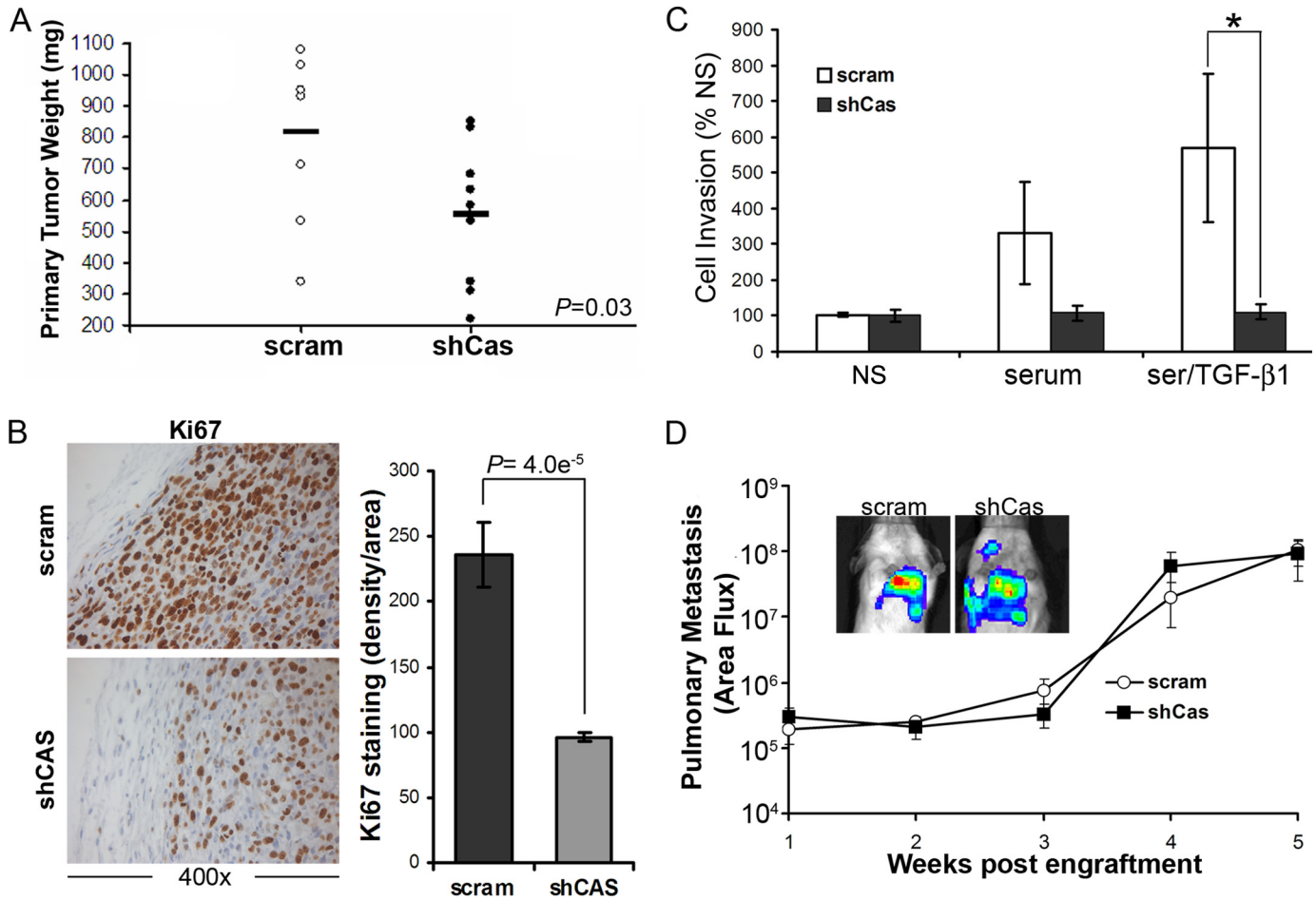

FIGURE 5. **p130Cas deficiency inhibits primary tumor growth and cell invasion.** *A*, control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells were engrafted onto the mammary fat pad of Balb/c mice. Primary tumors were removed surgically 21 days post-engraftment and weighed. *Bar* shows the mean tumor weights for each group (10 mice/group). $p = 0.03$. *B*, histological sections of primary tumors were stained with Ki67 to monitor the proliferative index of control (*scram*) and p130Cas-deficient (*shCas*) 4T1 tumors. Staining intensity was quantified over nine fields of view from three separate tumors/group and showed a decrease in the proliferative index at the invasive front of primary tumors upon p130Cas depletion. *C*, the invasion of control (*scram*) and p130Cas-deficient (*shCas*) 4T1 cells through Matrigel was stimulated by 2% fetal bovine serum (*serum*), or by 2% fetal bovine serum supplemented with TGF-β1 (5 ng/ml; *Ser/TGF-β1*). Data are the mean ± S.E. invasion to unstimulated MECs (*NS*) observed in three independent experiments completed in triplicate (*, $p < 0.05$). *D*, 4T1 cell derivatives were engrafted onto the mammary fat pads of female Balb/c mice as described in *A* and pulmonary photon flux readings were determined at the indicated time points post-engraftment. *Inset* shows representative bioluminescent signals of pulmonary metastases 4 weeks post-engraftment.

analysis of the affect p130Cas deficiency elicited on the activation of Smad2/3 by TGF-β in normal MECs. Despite this limitation, aberrant p130Cas expression has been associated with increased breast cancer progression and poorer clinical prognosis (18–20). Therefore, we sought to address the functional impact of p130Cas deficiency on the ability of TGF-β to initiate oncogenic signaling in the 4T1 metastatic model of breast cancer. Indeed, depletion of p130Cas greatly augmented the coupling of TGF-β to Smad2 and Smad3 in metastatic 4T1 cells without affecting total Smad2/3 levels (Fig. 3*A*). We previously demonstrated that the aberrant interaction of β3 integrin with TβR-II in post-epithelial-mesenchymal transition and malignant MECs is critical for the activation of p38 MAPK by TGF-β (11–13). Fig. 3*B* shows that p130Cas is not required for the formation of β3 integrin·TβR-II complexes in 4T1 cells, which serves to explain the slightly diminished coupling of TGF-β to p38 MAPK in p130Cas-depleted 4T1 cells (Fig. 3*A*). Furthermore, p130Cas deficiency not only elicited a significant increase in autocrine-driven SBE-luciferase activity in quiescent 4T1 cells (Fig. 3*C*), but also significantly augmented their induction of this Smad2/3-responsive reporter gene when stimulated by TGF-β (Fig. 3*C*). Accordingly, Smad2/3 localized primarily to the cytoplasm in quiescent parental 4T1 cells, as determined by (i) cellular fractionation coupled to Smad2/3 immunoblotting (Fig. 4*A*), and (ii) indirect Smad2/3 immunofluorescence (Fig. 4, *B* and *C*). In stark contrast, Smad2/3 was present in the cytoplasm and nuclear compartments in quiescent 4T1 cells that lacked p130Cas expression (Fig. 4, *A*–*C*), a finding consistent with their elevated basal levels of Smad2/3 activity. Moreover, p130Cas deficiency greatly accelerated the rate and extent of Smad2/3 that accumulated in the nucleus following TGF-β stimulation (Fig. 4, *A*–*C*). Collectively, these findings are consistent with the notion that aberrant p130Cas expression down-regulates the activity of Smad2/3 in metastatic breast cancer cells.

A characteristic phenotype of mammary carcinoma cells, including 4T1 cells, is their resistance to TGF-β-mediated growth arrest when grown in two-dimensional culture systems (12, 34, 35). Interestingly, the resultant increase in Smad2/3 activity elicited by p130Cas deficiency was unable to restore a

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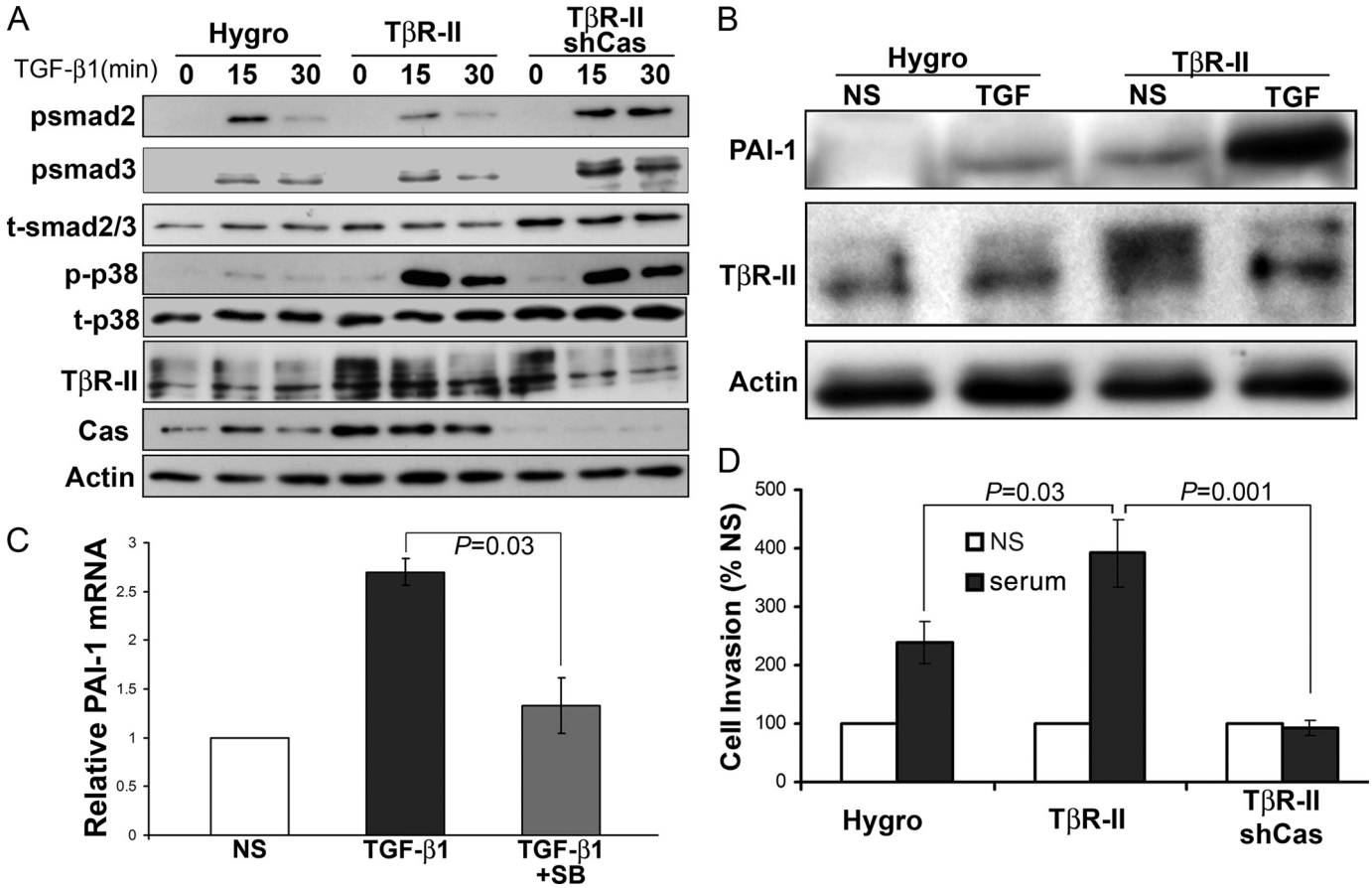

FIGURE 6. **p130Cas balances canonical and noncanonical TGF-β signaling.** *A,* control (*Hygro*), TβR-II- (*TβR-II*), or TβR-II-expressing 4T1 cells deficient in p130Cas expression (*TβR-II/shCas*) were stimulated with TGF-β1 (5 ng/ml) for varying times, and immunoblotted with phospho-specific antibodies against Smad2 (*psmad2*), Smad3 (*psmad3*), and p38 MAPK (*p-p38*) as indicated. Membranes were stripped and reprobed with antibodies against total Smad2/3 (*tSmad2/3*), p38 MAPK (*t-p38*), TβR-II, p130Cas (*Cas*), and β-actin (*Actin*) as loading controls. Data are from a representative experiment that was performed at least three times with similar results. *B,* quiescent control (*Hygro*) and TβR-II-expressing 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h. *NS,* no stimulation. The resulting conditioned medium was collected, precipitated, and immunoblotted for PAI-1. The corresponding cell lysates were probed for TβR-II and β-actin (*Actin*) as loading controls. Data are from a representative experiment that was performed three times with similar results. *C,* 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for 24 h in the absence or presence of the p38 MAPK inhibitor, SB208530 (10 μM), and analyzed by semi-quantitative reverse transcription-PCR for PAI-1 mRNA. Data are the mean ± S.E. induction of PAI-1 relative to unstimulated MECs (*NS*) observed in three independent experiments. *D,* 4T1-TβR-II cell variants described *A* were induced to invade synthetic basement membranes by 2% fetal bovine serum (*serum*). Data are the mean ± S.E. invasion relative to unstimulated (*NS*) controls of three independent experiments completed in triplicate.

strong cytostatic response in 4T1 cells upon TGF-β administration (see supplemental Fig. S2*A*). However, it is known that culturing cells on plastic can mask several cell signaling events, most notably those of TGF-β (31, 36, 37). As such, we propagated control and p130Cas-depleted 4T1 cells in three-dimensional organotypic cultures in the absence or presence of TGF-β1 (Fig. 4*D*). In addition to restoring a more rounded, normal acinar structure (Fig. 4*D*), depletion of p130Cas also significantly increased the growth inhibitory effects of TGF-β1 as compared their p130Cas-expressing counterparts (Fig. 4*E*). Taken together, these findings suggest that p130Cas functions to sequester Smad2/3 in the cytoplasm, thereby inhibiting their activity and the cytostatic function of TGF-β. Our findings also suggest that aberrant expression of p130Cas may elicit profound effects on mammary tumor growth regulated by TGF-β.

p130Cas Deficiency Inhibits Primary Mammary Tumor Growth and Cell Invasion—To further assess the role of p130Cas in TGF-β-mediated tumor progression, we engrafted parental and p130Cas-deficient 4T1 cells onto the mammary fat pads of syngeneic Balb/c mice. Indeed, orthotopic tumors lacking p130Cas clearly grew more slowly as compared with their parental counterparts (Fig. 5*A*). Consistent with a reduction in 4T1 tumor weights, we also observed p130Cas deficiency to elicit significantly impaired proliferative indices as determined by Ki67 immunohistochemistry of primary tumor sections (Fig. 5*B*). These data support our *in vitro* findings (Figs. 2*D* and 4*E*) and suggest that measures capable of inactivating p130Cas expression and/or function may provide a novel means to partially restore the tumor suppressive activity of TGF-β.

Through its inclusion in focal adhesion complexes, p130Cas has also been proposed to play a critical role in mediating cell migration and invasion (15). Indeed, p130Cas deficiency abrogated 4T1 cell invasion induced by TGF-β1 (Fig. 5*C*); however, this same cellular condition failed to impact the pulmonary metastasis of 4T1 cells engrafted onto the mammary fat pad of Balb/c mice (Fig. 5*D*). These findings underscore the complexities of carcinoma metastasis *in vivo* and point to the existence of alternative and TGF-β-independent pathways that can compensate for the loss of cellular invasion normally regulated by

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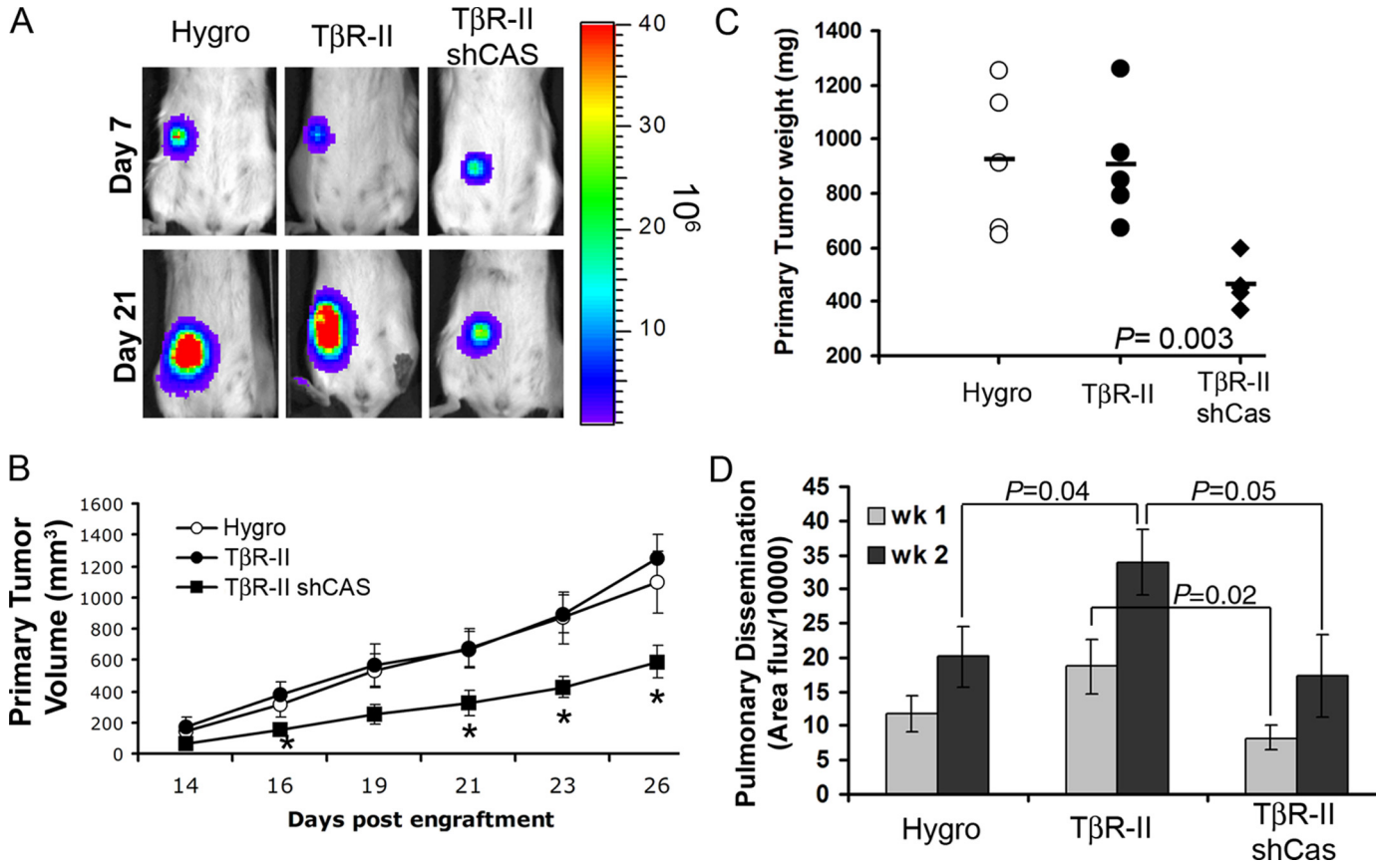

FIGURE 7. **Coupling TβR-II expression with p130Cas deficiency prevents TGF-β-driven breast cancer metastasis.** *A*, control (*Hygro*), TβR-II- (*TβR-II*), and TβR-II-expressing 4T1 cells lacking expression of p130Cas (*TβR-II/shCas*) were engrafted onto the mammary fat pad of Balb/c mice. Bioluminescent visualization of primary 4T1 tumors showed an equal establishment at day 7 post-engraftment, but a significant growth defect at day 26 in TβR-II-expressing 4T1 tumors lacking p130Cas expression. *B*, data are the mean ± S.E. of tumor volumes measured for the indicated 4T1 tumor variants. *, $p < 0.05$, $n = 5$ mice/group. *C*, primary 4T1 tumors were removed surgically 26 days post-engraftment and weighed. *Bar* shows the mean tumor weights for each group (5 mice/group), $p = 0.003$. *D*, data are the mean ± S.E. ($n = 5$ mice/group) of pulmonary photon flux units measured at 1-week intervals following engraftment of the 4T1 variants onto the mammary fat pads.

p130Cas. Indeed, it is tempting to speculate that pulmonary metastasis of p130Cas-deficient 4T1 cells reflects their maintenance of p38 MAPK activity (Fig. 3*A*), a signaling pathway in which we demonstrated previously to be necessary for TGF-β stimulation of 4T1 pulmonary metastasis (11).

p130Cas Balances Canonical and Noncanonical TGF-β Signaling—We previously demonstrated that transgenic expression of human TβR-II in 4T1 cells significantly enhances their invasion (12) and pulmonary metastasis (11) in mice. Therefore, we next sought to utilize this model to specifically address the role of p130Cas in mediating TGF-β-driven tumor progression and metastasis. Indeed, transgenic expression of TβR-II dramatically enhanced the coupling of TGF-β to the activation of p38 MAPK, but had little to no affect on Smad2 or Smad3 phosphorylation (Fig. 6*A*). This shift in TGF-β signaling was reflected by the increased basal and TGF-β-induced expression of the prometastatic factor, plasminogen activator inhibitor-1 (PAI-1; Fig. 6*B*). Moreover, pharmacological inhibition of p38 MAPK activity significantly impaired the ability of 4T1 cells to up-regulate PAI-1 in response to TGF-β1 (Fig. 6*C*). Importantly, rendering these "hyperinvasive" 4T1-TβR-II cells deficient in p130Cas had no appreciable effect on their enhanced ability to phosphorylate p38 MAPK in response to TGF-β; however, this same cellular condition did elicit elevated phosphorylation of both Smad2 and Smad3 (Fig. 6*A*). Thus, diminishing p130Cas expression in 4T1-TβR-II cells restores the physiologic balance between canonical and noncanonical TGF-β signaling (Fig. 6*A*). In accord with their increased p38 MAPK activity and PAI-1 secretion, 4T1-TβR-II cells are significantly more invasive compared with parental 4T1 cells (Fig. 6*C*) (11, 12). Importantly, depleting 4T1-TβR-II cells of p130Cas expression abrogated their enhanced invasiveness mediated by TβR-II expression and a serum stimulation (Fig. 6*C*). Taken together, these findings clearly show that elevated TβR-II expression enhances the coupling of TGF-β to its noncanonical effector, p38 MAPK, leading to augmented PAI-1 expression and cellular invasion. Furthermore, we show for the first time that p130Cas deficiency restores a physiologic balance between canonical and noncanonical TGF-β signaling, and as such, prevents breast cancer cell invasion.

p130Cas Deficiency Prevents Early TGF-β-driven Breast Cancer Dissemination—We next sought to utilize the 4T1-TβR-II model to define the specific role of p130Cas in mediating *in vivo* TGF-β-driven breast cancer progression. Bioluminescent imaging of tumor bearing Balb/c mice showed that parental, TβR-II-, and TβR-II-shCas-expressing 4T1 tumors exhibited similar rates of establishment (Fig. 7*A*, *Day 7*). However, their growth rates thereafter diverged rapidly due to the

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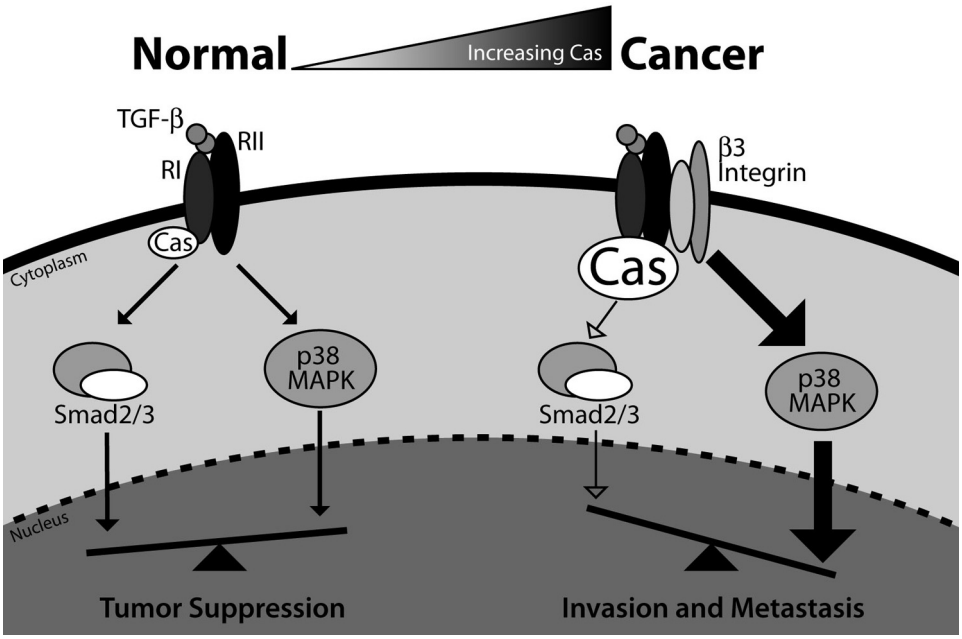

FIGURE 8. **p130Cas functions as a molecular rheostat that maintains the balance between canonical and noncanonical TGF-β signaling.** In normal MECs, physiologic expression levels of integrins and p130Cas maintain the balance between Smad2/3 and p38 MAPK signaling, which collectively support the tumor suppressing and cytostatic functions of TGF-β. During breast cancer progression, aberrant up-regulation of p130Cas expression inhibits Smad2/3 activation in a manner that parallels the inappropriate formation of β3 integrin·TβR-II complexes, which promotes increased coupling of TGF-β to p38 MAPK. Overall, these untoward events shift the balance of TGF-β signaling to favor activation of noncanonical effectors, particularly p38 MAPK, during the acquisition of metastatic phenotypes by breast cancer cells. Importantly, rendering late-stage breast cancer cells deficient in p130Cas enhances the activation of Smad2/3 by TGF-β, which thereby restores its ability to suppress the growth and pulmonary metastasis of breast cancer cells in mice.

inability of TβR-II-shCas tumors to grow out as efficiently as the parental and TβR-II-expressing control cells (Fig. 7, *A* and *B, Day 21*). Importantly, combining TβR-II expression with p130Cas depletion significantly exacerbated (by 10-fold) the growth defects originally observed upon p130Cas depletion in wild-type 4T1 tumors (Fig. 7*C*). Thus, abrogating p130Cas was sufficient in restoring the tumor suppressing activities of TGF-β.

Finally, we found that TβR-II expression elicited a dramatic increase in the early dissemination of 4T1 tumors to lungs as compared with parental cells (Fig. 7*D*). Importantly, this TGF-β/TβR-II-driven metastatic process was specifically inhibited by rendering TβR-II-expressing 4T1 cells deficient in p130Cas (Fig. 7*D*). Taken together, these findings show that p130Cas is critically involved in promoting primary mammary tumor growth, and is specifically required in facilitating early events in TGF-β-driven primary tumor dissemination.

DISCUSSION

TGF-β is a principal player involved in suppressing mammary tumorigenesis, doing so through its ability to maintain the composition of normal MEC microenvironments, and by inhibiting the aberrant proliferation of normal MECs (6, 38). Mammary tumorigenesis has evolved a variety of mechanisms that subvert the tumor suppressing functions of TGF-β, and in doing so, confer oncogenic and metastatic activities upon this multifunctional cytokine (34). Indeed, how TGF-β both suppresses and promotes mammary tumorigenesis remains a fundamental question that directly impacts the ability of science and medicine to effectively target the TGF-β signaling system during the treatment of breast cancer patients. Deciphering this paradox remains the most important question concerning the biological and pathological actions of this multifunctional cytokine (39).

We previously established the importance of aberrant interactions between β3 integrin and TβR-II to promote Src-mediated phosphorylation of TβR-II, which then recruits and binds Grb2. Once bound to phospho-Tyr-284 in TβR-II, Grb2 facilitates TGF-β-mediated activation of noncanonical MAP kinase signaling without affecting the coupling of TGF-β to Smad2/3 (12, 13). Importantly, measures capable of disrupting this signaling axis readily prevent TGF-β from driving breast cancer invasion and metastasis (11, 40). Thus, in addition to establishing the critical importance of p38 MAPK activation in mediating breast cancer metastasis stimulated by TGF-β, these studies also suggested that inappropriate imbalances between canonical and noncanonical TGF-β signaling systems may in fact underlie its prometastatic activities in breast cancer cells. Our findings herein provide the first definitive evidence that (i) canonical and noncanonical signaling imbalances do indeed dictate MEC response to TGF-β, and (ii) p130Cas functions as a novel molecular rheostat that governs the delicate balance between canonical and noncanonical TGF-β effectors. Indeed, overexpression of either full-length or the carboxyl terminus of p130Cas was sufficient to decrease TGF-β-induced Smad2/3 phosphorylation while simultaneously increasing that of p38 MAPK. Moreover, depleting p130Cas significantly increased the activity of Smad2/3 and concomitantly decreased that of p38 MAPK induced by TGF-β, and finally, elevating TβR-II expression amplified the activation of p38 MAPK by TGF-β, which significantly enhanced early metastatic progression of mammary tumors in mice (Fig. 7) (11). In "hypermetastatic" TβR-II-expressing cells, p130Cas deficiency similarly increased the coupling of TGF-β to Smad2/3, an event that negated the proinvasive and prometastatic activities of p38 MAPK in developing 4T1 tumors. Thus, p130Cas functions in balancing the activation status of canonical and noncanonical effectors targeted by TGF-β, findings that are clinically and medically relevant to the development and progression of mammary tumors regulated by TGF-β.

A schematic depicting the function of p130Cas in TGF-β signaling is presented in Fig. 8. Indeed, in normal MECs, TGF-β receptors fail to interact significantly with integrins, which lim-

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its TGF-β stimulation of p38 MAPK and the initiation of oncogenic signaling by TGF-β (13, 40–42). The net effect of these signaling events results in tumor suppression by TGF-β. However, during mammary tumorigenesis, p130Cas expression is up-regulated dramatically, as is the aberrant formation of integrin and TGF-β receptor complexes (11–13, 40), which collectively decrease the activity of Smad2/3 and increase that of p38 MAPK and other noncanonical effectors that promote breast cancer metastasis stimulated by TGF-β (11, 40). This signaling imbalance can be potentiated by elevated TβR-II expression and its consequential enhancement of p38 MAPK activation and metastasis (11, 34, 40, 43). In all cases, these various signaling inputs are critically balanced and influenced by the level of p130Cas expression. Indeed, we (see Fig. 1) and others (20) find mammary tumorigenesis to dramatically increase the expression of p130Cas. Based on our findings presented herein, we suggest that this event limits TGF-β stimulation of Smad2/3, which (i) diminishes MEC responsiveness to the cytostatic activities of TGF-β (44); and (ii) promotes amplified coupling of TGF-β to its noncanonical effectors, leading to breast cancer invasion and metastasis. In fact, our findings strongly support the progressive hypothesis that inappropriate imbalances between canonical and noncanonical TGF-β signaling systems underlies the acquisition of metastatic phenotypes in mammary carcinomas, as well as facilitates the oncogenic switch of TGF-β from a tumor suppressor to a prometastatic molecule.

Along these lines, a recent report suggests that murine mammary tumor virus-driven p130Cas expression in mice is sufficient to induce mammary gland hyperplasia (20). However, it was necessary to combine transgenic p130Cas expression with that of HER2 to enhance formation of mammary tumors (20). Although specific effects on TGF-β activity and signaling were not examined in this mouse model, these findings do suggest that the tumor promoting properties of p130Cas only manifest in the face of additional oncogenic signaling inputs (*i.e.* elevated HER2 expression), which mirrors our own results showing that heightened TGF-β signaling (TβR-II expression) requires p130Cas to induce pulmonary dissemination. Moreover, we show that transgenic TβR-II expression led to increased basal and TGF-β-induced production of the prometastatic protein, PAI1, without impacting the phosphorylation of Smad2/3. These findings suggest that (i) p130Cas specifically regulates the activity of Smad2/3 as opposed to that of the TGF-β receptors, and (ii) Smad2/3 expression levels, not those of TGF-β receptors, are rate-limiting during the activation of canonical TGF-β signaling. Thus, p130Cas acts as a molecular rheostat of canonical Smad2/3 and noncanonical p38 MAPK signaling stimulated by TGF-β, and disruption of the balance between these two pathways has dramatic affects on breast cancer growth and progression.

In summary, we demonstrated that p130Cas functions to regulate the balance between TGF-β-mediated activation of Smad2/3 and p38 MAPK in normal and metastatic MECs. Moreover, we provide compelling evidence that p130Cas is both necessary and sufficient to drive the oncogenic activities of TGF-β, including its regulation of mammary tumor growth and the initiation of early steps in the metastatic dissemination of breast cancer cells. Collectively, our findings establish p130Cas as an essential mediator that underlies the oncogenic conversion of TGF-β function, thereby enhancing its ability to promote the progression of mammary carcinomas.

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Supplemental Figures

p130Cas IS REQUIRED FOR MAMMARY TUMOR GROWTH AND TRANSFORMING GROWTH FACTOR-β (TGF-β)-MEDIATED METASTASIS THROUGH REGULATION OF SMAD2/3 ACTIVITY

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Supplemental Figures:

Figure S1: M.K. Wendt, J.A. Smith, and W.P. Schiemann

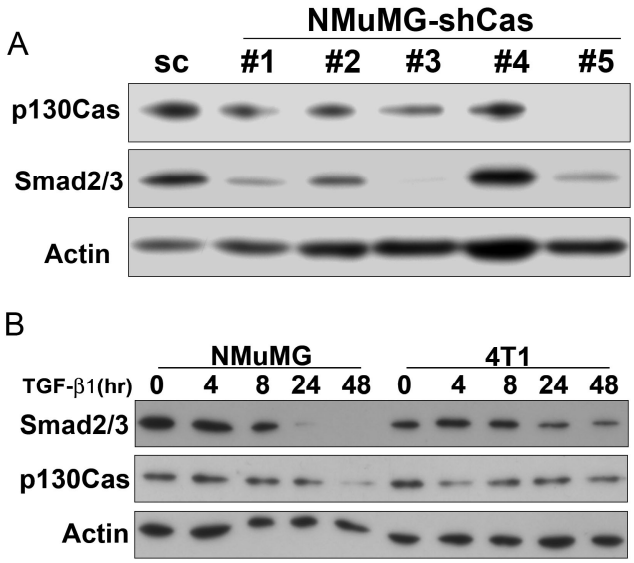

FIGURE S1. **p130Cas-deficiency decreases Smad2 expression in normal MECs.** *A*, Five unique shRNA sequences targeting p130Cas (shCas #1-5) were stably expressed in NMuMG cells, and differences in p130Cas expression were analyzed by immunoblotting with anti-p130Cas antibodies (sc, scrambled shRNA). β-actin immunoreactivity (Actin) is provided as a loading control. *B*, Quiescent NMuMG and 4T1 cells were stimulated with TGF-β1 (5 ng/ml) for varying times, and the resulting whole-cell extracts were immunoblotted with Smad2/3 antibodies, followed by p130Cas and β-actin (Actin) as a loading control. Data are representative of 2 independent experiments and show that Smad2/3 are degraded more readily upon prolonged TGF-β treatment in NMuMG cells as compared to 4T1 cells.

Figure S2: M.K. Wendt, J.A. Smith, and W.P. Schiemann

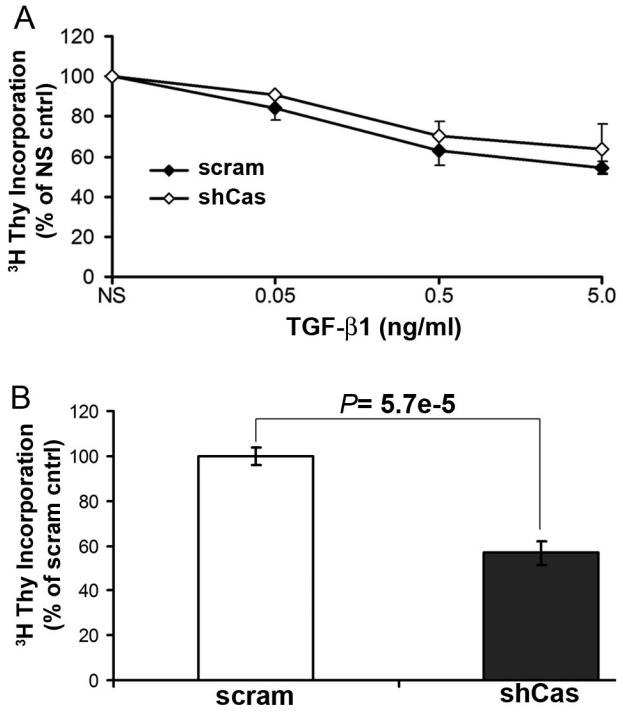

FIGURE S2. **p130Cas-deficiency decreases basal 4T1 proliferation but does not affect TGF-β1-growth inhibition when cultured on plastic**. *A*, Control (scram) and p130Cas-deficient (shCas) cells were stimulated with the increasing TGF-β1 concentrations for 48 h as indicated. Afterward, [^{3}H]thymidine incorporation into cellular DNA was determined. Data are the mean (± SE; n=3) [^{3}H]thymidine incorporation normalized to untreated controls (NS, no stimulation). *B*, Data are the mean (± SE; n=3) basal [^{3}H]thymidine incorporation normalized to control (scram) 4T1 cells.

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The Pathophysiology of Epithelial-Mesenchymal Transition Induced by Transforming Growth Factor-β in Normal and Malignant Mammary Epithelial Cells

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Abstract Epithelial-mesenchymal transition (EMT) is an essential process that drives polarized, immotile mammary epithelial cells (MECs) to acquire apolar, highly migratory fibroblastoid-like features. EMT is an indispensable process that is associated with normal tissue development and organogenesis, as well as with tissue remodeling and wound healing. In stark contrast, inappropriate reactivation of EMT readily contributes to the development of a variety of human pathologies, particularly those associated with tissue fibrosis and cancer cell invasion and metastasis, including that by breast cancer cells. Although metastasis is unequivocally the most lethal aspect of breast cancer and the most prominent feature associated with disease recurrence, the molecular mechanisms whereby EMT mediates the initiation and resolution of breast cancer metastasis remains poorly understood. Transforming growth factor-β (TGF-β) is a multifunctional cytokine that is intimately involved in regulating numerous physiological processes, including cellular differentiation, homeostasis, and EMT. In addition, TGF-β also functions as a powerful tumor suppressor in MECs, whose neoplastic development ultimately converts TGF-β into an oncogenic cytokine in aggressive late-stage mammary tumors. Recent findings have implicated the process of EMT in mediating the functional conversion of TGF-β during breast cancer progression, suggesting that the chemotherapeutic targeting of EMT induced by TGF-β may offer new inroads in ameliorating metastatic disease in breast cancer patients. Here we review the molecular, cellular, and microenvironmental factors that contribute to the pathophysiological activities of TGF-β during its regulation of EMT in normal and malignant MECs.

Keywords Epithelial-mesenchymal Transition · Metastasis · Signal transduction · Transforming growth factor-β · Tumor microenvironment

Abbreviations

α-SMA	α-smooth muscle actin
BMP	Bone morphogenic protein
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
JNK	c-Jun N-terminal kinase
MAP kinase	Mitogen-activated protein kinase
MEC	Mammary epithelial cell
mTOR	Mammalian target of rapamycin
MET	Mesenchymal-epithelial transition
MTA3	Metastasis associated protein 3
NF-κB	Nuclear factor-κB
PAI	Plasminogen activator inhibitor
PDGF	Platelet-derived growth factor
PI3K	Phosphoinositide-3-kinase
TβR-I	TGF-β type I receptor
TβR-II	TGF-β type II receptor
TβR-III	TGF-β type III receptor

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TGF-β	Transforming growth factor-β
uPA	Urokinase plasminogen activator
uPAR	uPA receptor
ZO-1	Zonula occluden-1

Introduction

Epithelial-mesenchymal transition (EMT) is a complex process whereby polarized epithelial cells transition into apolar fibroblastoid-like cells, a phenomenon that underlies tissue morphogenesis and organogenesis in the embryo, as well as tissue remodeling and repair in adults [1–3]. Moreover, the inappropriate reactivation of developmental EMT programs plays a significant role in the pathology of fibrotic diseases and cancer, including those of the breast. Epithelial cell sheets manifest as tightly packed cell monolayers that compose the skin and line the internal cavities (e.g., airways and gastrointestinal tract), and in doing so, form a barrier that protects the host from environmental insults. In a similar fashion, mammary epithelial cells (MECs) exhibit a cobblestone appearance and are linked through the actions of numerous cell–cell complexes, including desmosomes, adherens, gap, and tight junctions [4]. Collectively, these junctional structures provide MECs with their characteristic apical-basolateral polarity and cortical actin architecture. In stark contrast, mesenchymal cells lack cell–cell junctional complexes, leading to their apolar morphologies and enhanced migratory activities through the extracellular matrix (ECM). The plasticity of MECs enables them to dedifferentiate during EMT, and in doing so, transitioning MECs forego their cobblestone morphologies and instead acquire a spindle-shaped appearance characteristic of mesenchymal cells. In undertaking this phenotypic and morphologic transition, MECs first experience a disruption and delocalization of tight junction complexes (e.g., zonula occluden-1 (ZO-1), claudin, and occludin), which is succeeded by the loss of E-cadherin expression and activity that results in the stabilization and nuclear accumulation of β-catenin. This process is also characterized by the dramatic remodeling of the cytoskeleton and its formation of actin stress fibers as transitioning cells acquire migratory mesenchymal phenotypes [1–3]. Thus, EMT reflects the initiation of a complex cascade of genetic and epigenetic events that culminate in MECs discarding their expression of epithelial gene signatures (e.g., E-cadherin, β4 integrin, and ZO-1) and acquiring those of mesenchymal cells [e.g., N-cadherin, vimentin, α-smooth muscle actin (α-SMA)]. Moreover, the process of EMT is highly metastable and is readily subject to phenotypic and morphologic reversion by mesenchymal-epithelial transition (MET), the molecular mechanisms of which are poorly understood and will not be discussed further herein (*see* [5, 6]). These general steps exhibited by transitioning MECs underlie both the biological and pathological episodes of EMT, which recently have been categorized into three distinct subtypes—i.e., *a*) embryonic and developmental EMT, which is referred to as type 1 EMT; *b*) tissue regeneration and fibrotic EMT, which is referred to as type 2 EMT; and *c*) cancer progression and metastatic EMT, which is known as type 3 EMT [2].

Here we review recent findings that directly impact our understanding of the role transforming growth factor-β (TGF-β) plays in regulating the initiation and resolution of individual subtypes of EMT. In addition, we also discuss the clinical implications afforded by chemotherapeutic targeting of TGF-β effectors coupled to type 3 EMT and their potential to suppress breast cancer progression and the oncogenic activities of TGF-β, particularly its induction of EMT and metastasis in developing mammary carcinomas.

TGF-β and EMT Subtypes

TGF-β Signaling and Epithelial Plasticity

TGF-β is a multifunctional cytokine and a powerful tumor suppressor that governs essentially every aspect of the physiology and homeostasis of MECs, including their ability to proliferate, migrate, differentiate, and survive [7–9]. During mammary tumorigenesis, a variety of genetic and epigenetic events conspire to circumvent the cytostatic and tumor suppressing activities of TGF-β, thereby enhancing the development and progression of evolving mammary neoplasms [1, 7, 8]. Even more remarkably, neoplastic MECs that have acquired resistance to the cytostatic activities of TGF-β often exhibit oncogenic behaviors when stimulated by TGF-β. This phenotypic switch in TGF-β function during tumorigenesis is known as the "TGF-β Paradox," which represents the most important and unanswered question concerning the pathophysiological actions of this pleiotropic cytokine [10]. Interestingly, the differentiation and migration of mammary stem cells results in the production of both the outer myoepithelial and inner luminal layers that ultimately give rise to mature mammary glands [11–13], suggesting that the process of EMT is in someway linked to the generation and maintenance of stem cell populations. Numerous studies have established TGF-β as a master regulator of EMT in normal and malignant MECs [1, 14, 15], while more recent findings have associated TGF-β stimulation of EMT with the acquisition of "stemness" in transitioning MECs [16, 17], and with the selection and expansion of breast cancer stem cells [18, 19]. Along these lines, we and others have established EMT as being a vital component underlying the

initiation of oncogenic TGF-β signaling in normal and malignant MECs [1, 14, 20]. Thus, identifying the molecular mechanisms whereby EMT is induced by TGF-β is paramount to maintaining mammary gland homeostasis, and to suppressing the development and progression of mammary tumors.

The mechanisms through which TGF-β initiates its pathophysiological activities and initiation of EMT are shown schematically in Fig. 1. Indeed, transmembrane signaling stimulated by TGF-β commences by its binding to the high-affinity transmembrane Ser/Thr receptor protein kinases, TGF-β type I (TβR-I) and type II (TβR-II). Mammals express three distinct TGF-β isoforms termed TGF-β1, TGF-β2, and TGF-β3, which function analogously in vitro, but give rise to more than 30 distinct phenotypes upon their genetic deletion in mice [21]. In addition, whereas TGF-βs 1 and 3 can bind directly to TβR-II, TGF-β2 requires assistance from the accessory TGF-β receptor, TGF-β type III (TβR-III). In all of these scenarios, the ligation of TGF-β to TβR-II facilitates its transphosphorylation and activation of TβR-I, which subsequently phosphorylates and activates the latent transcription factors, Smads 2 and 3. Once phosphorylated, receptor-activated Smads 2 and 3 associate with the common Smad, Smad4, at which point these heteromeric complexes translocate en masse into the nucleus to regulate the expression of TGF-β-responsive genes. In addition, the amplitude and duration of Smad2/3-based signaling transpires through their physical interaction with a plethora of transcription factors, and with a variety of transcriptional activators and co-repressors in a gene- and cell-specific manner (*see* [22, 23]). The capacity of Smad2/3 to impact MEC behavior is also governed by their association with a number of adapter molecules, including SARA [24], Hgs [25], and Dab2 [26–28]. Likewise, upregulated expression of the inhibitory Smad, Smad7, also limits the extent of Smad2/3 signaling by competitively inhibiting their phosphorylation by TβR-I [29–31], and by promoting the internalization and degradation of TβR-I [32, 33]. Moreover, the anti-TGF-β activity of Smad7 is augmented by its interaction with the adaptor protein STRAP [34], and conversely, is attenuated by its association with either AMSH2 [35] or Arkadia [36–38]. Collectively, TGF-β signals propagated through Smad2/3 are referred to as "canonical" TGF-β signaling, and their specific role in regulating EMT induced by TGF-β is discussed below.

Besides its ability to activate canonical Smad2/3-dependent pathways, TGF-β also regulates MEC behavior and the induction of EMT via the stimulation of numerous "non-canonical" effector systems, including (*a*) small GTP-binding proteins (e.g., Ras, Rho, and Rac1); (*b*) phosphoinositide-3-kinase (PI3K), AKT, and mTOR; (*c*) MAP kinases (e.g., p38 MAPK, ERK1/2, and JNK); and (*d*) NF-κB and Cox-2 [39–51]. In addition, although inactivating mutations and decreased expression of TβR-I and TβR-II have been identified and characterized in human cancers, the occurrence of these mutagenic events is in fact quite rare. However, the loss of TβR-III expression does correlate with increased breast cancer tumorigenicity and decreased patient survival [52, 53], suggesting an important tumor suppressing function for this accessory receptor. Indeed, the functional loss of TβR-III coincides with EMT and enhances cell migration and invasion [54, 55]. The coupling of TGF-β to these receptors and their noncanonical effectors is depicted schematically in Fig. 1, as is our understanding of how integrins and proteins associated with focal adhesion complexes cooperate with TGF-β in regulating the behaviors and EMT status of normal and malignant MECs [45, 46, 56–60]. The specific role of these noncanonical TGF-β effectors in regulating EMT in normal and malignant MECs is presented in the succeeding sections, while future studies clearly need to address the relative contribution of TβR-I, TβR-II, and TβR-III in mediating activation of canonical and non-canonical TGF-β signaling systems.

EMT Subtypes Coupled to TGF-β

The process of EMT occurs in a variety of distinct biological and pathological settings, including during normal embryogenesis and tissue morphogenesis, during tissue remodeling and repair, and during fibrosis and cancer progression. Although the underlying molecular mechanisms that define the pathophysiological activities of EMT in distinct cellular contexts are likely to be overlapping and redundant, the diversity of biological outcomes engendered by EMT is nonetheless highly specialized and has resulted in the classification of three distinct subtypes of EMT [2]. For instance, type 1 EMT is activated during embryogenesis and tissue morphogenesis, leading to the generation of mesenchymal cells that ultimately give rise to secondary epithelial structures. In contrast, type 2 EMT is normally activated during tissue regeneration and repair, and abnormally during tissue fibrosis resulting from dysregulated inflammatory reactions. Finally, type 3 EMT is activated by cancer cells, including those of the breast [1], to facilitate their acquisition of invasive and metastatic phenotypes, and ultimately to establish secondary sites of lethal tumor outgrowth. In the succeeding sections, we highlight the molecular mechanisms and biological settings whereby TGF-β participates in regulating individual EMT subtypes, particularly type 3 EMT during mammary tumorigenesis.

Type 1 EMT

Developmental or type 1 EMT is associated with embryogenesis and its accompanying organogenesis and tissue morphogenesis, both of which require epithelial-derived

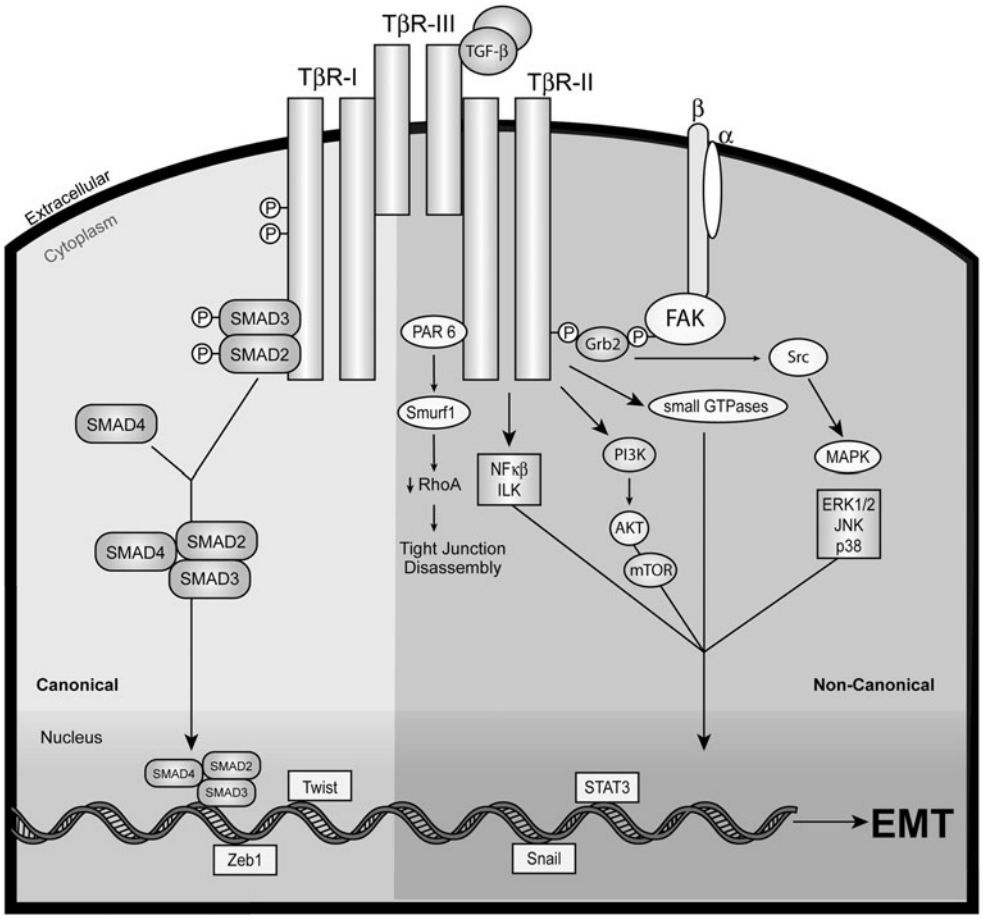

Figure 1 Schematic depicting the canonical and noncanonical TGF-β signaling systems coupled to EMT in MECs. Transmembrane signaling by TGF-β ensues through its binding and activation of the Ser/Thr protein kinase receptors, TβR-I and TβR-II. Indeed, TGF-β binding to either TβR-III or TβR-II enables the recruitment and transphosphorylation of TβR-I, resulting in its activation and subsequent phosphorylation of the receptor-activated Smads, Smad2 and Smad3. Once activated, Smad2/3 form heterocomplexes with the common Smad, Smad4, which collectively translocate en masse to the nucleus to regulate the expression of TGF-β-responsive genes in concert with an ever expanding list of transcriptional coactivators and repressors. This branch of the bifurcated TGF-β signaling system represents the "canonical" or "Smad2/3-dependent" TGF-β pathway (*left*). Alternatively, TGF-β also activates a variety of "noncanonical" or "Smad2/3-independent" effectors, including Par6, NF-κB, ILK, FAK, Src, a variety of small GTPases, members of the MAP kinase family (e.g., ERK1/2, JNK, and p38 MAPK), and the PI3K:AKT:mTOR signaling axis (*right*). Activation of members of the Snail, ZEB, or bHLH family of transcription factors promote EMT by transcriptionally repressing the production of E-cadherin transcripts (*bottom*). Imbalances between canonical and noncanonical TGF-β signaling pathways have recently been identified and established as casual aberrancies underlying the initiation of oncogenic TGF-β signaling and its initiation of EMT in normal and malignant MECs. See text for details.

mesenchymal cells to undergo MET during the formation of new epithelial structures (Fig. 2). In stark contrast to types 2 and 3 EMT, this developmental mode of EMT results in the production of multipotent mesenchymal cells and is not associated with tissue fibrosis and inflammation, or with the aberrant migration of cancer cells. Interestingly, members of the TGF-β superfamily play important regulatory roles during type 1 EMT, particularly during the processes of gastrulation, palate fusion, and neural crest and endocardial cushion formation [61]. Indeed, type 1 EMT is first initiated during embryogenesis to promote gastrulation, which results in the formation of the ectoderm, mesoderm, and endoderm from the invaginating primitive streak [2, 4]. This process requires Wnt signaling inputs to render primitive streak cells competent to undergo EMT in response to the TGF-β superfamily members, Nodal and Vg1 [62–65], both of which are essential for primitive streak and mesoderm formation in gastrulating embryos [66–70].

Type 1 EMT is also initiated during neurulation and occurs in the neural plate to facilitate the formation of the neural tube, which ultimately gives rise to the spine and brain. As the neural tube fuses, EMT also occurs in neural crest cells and facilitates their migration and dissemination throughout the embryo where they ultimately contribute to the generation of numerous specialized tissues, including

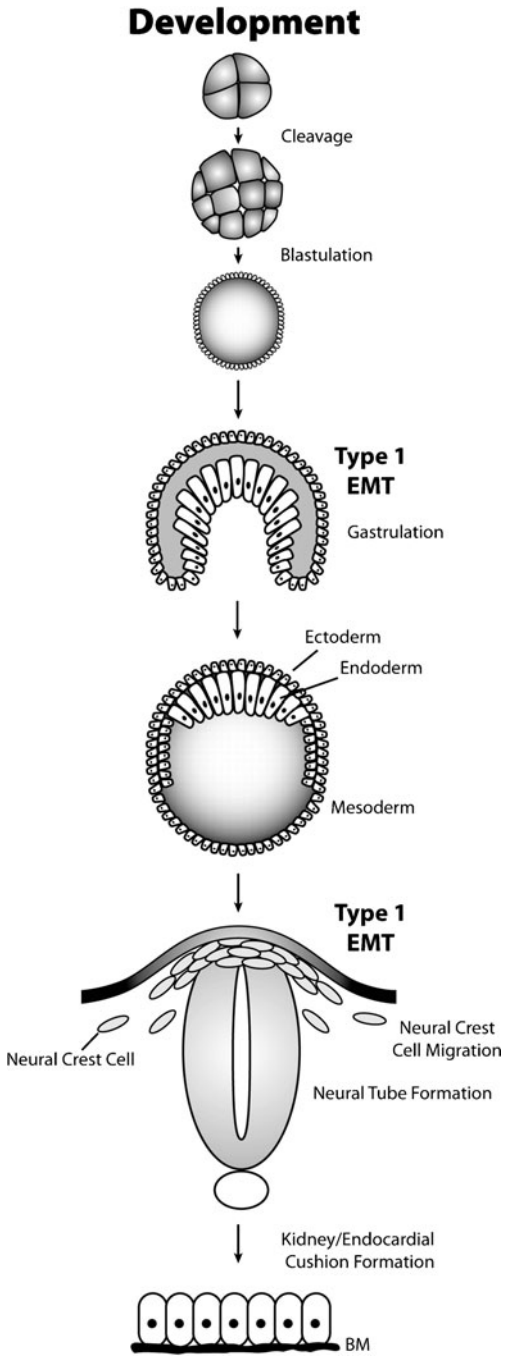

Figure 2 Type 1 EMT in development and embryogenesis. Following fertilization, the zygote undergoes several rounds of cleavage to form a dense spheroid structure, which then undergoes blastulation to form a hollowed blastula. During gastrulation, TGF-β provides inductive EMT signals that elicits the formation of an invaginating structure that generates the three multipotent germ layers, namely the mesoderm, endoderm, and ectoderm. The mesoderm gives rise to muscle, skeleton, and connective tissue, while the endoderm gives rise to internal organs, such as the liver, colon, and stomach. The outer ectodermal layer generates the epidermis and ocular lens, as well as produces the mammary gland. The ectoderm also gives rise to the primordial nervous system via neurulation and neural crest formation, a process that is dependent upon EMT induced by TGF-β. Finally, TGF-β stimulation of EMT during organogenesis is essential for the faithful development of the kidney, and of the endocardial cushion and subsequent atrioventricular valve formation. The role of TGF-β in regulating type 1 EMT during mammary gland branching and invasion is discussed in the text.

the adrenal medulla and the peripheral nervous and skeletal systems [4, 71]. Bone morphogenic proteins (BMPs) belong to the TGF-β superfamily and are essential to the induction of EMT during neurulation [72, 73]. Similarly, TGF-β signaling also oversees the latter stages of heart and secondary palate formation [4, 74]. For instance, myocardial cells actively secrete an ECM that separates the endocardium from the myocardium, which also induces endocardial cells to undergo EMT and migrate into the endocardial cushion to facilitate atrioventricular valve formation [4]. Experiments performed in chicks and mice have identified important roles during development for all three TGF-β isoforms, of which TGF-β2 appears to play a dominant role in stimulating EMT in endocardial cells [75–77]. Similarly, TGF-β3 is essential in mediating secondary palate formation, which requires midline epithelial seam cells to undergo EMT to complete oral palate fusion and closure [78, 79]. Importantly, TβR-III expression is essential for EMT induced during the formation of both the heart and secondary palate [80, 81].

At present, a role for type 1 EMT in mediating mammary gland development has yet to be described; however, branching morphogenesis of normal MEC organoids in 3D-organotypic cultures showed that these structures exhibit a loss in polarity and acquire mesenchymal characteristics at invading branch tips, findings that point to a role of type 1 EMT in mammary gland development [82, 83]. In addition, inappropriate reactivation of embryonic and type 1 EMT programs have been associated with the development and progression of mammary tumors [61]. Indeed, aberrantly elevated expression of the Six1 homeoprotein not only elicits the formation of mammary tumors [84], but also stimulates breast cancer EMT and metastasis in part via a TGF-β-dependent mechanism [85]. Thus, thoroughly defining the role of TGF-β during type 1 EMT will be critical to the development of novel chemotherapeutics capable of circumventing these activities during the inappropriate reactivation of type 1 EMT programs in mammary tumors.

Type 2 EMT

The initiation of type 2 EMT is essential in maintaining tissue homeostasis through its ability to induce wound healing and tissue remodeling in response to noxious insults. An important distinction between type 1 and type 2 EMT is that the latter is governed by inflammatory reactions, whose cessation resolves the EMT phenotype following wound repair [2]. A

corollary states that chronic inflammation underlies the initiation of tissue and organ fibrosis, which eventually elicits organ dysfunction and destruction if left unabated. As depicted in Fig. 3, the process of wound healing involves the orchestrated activities of numerous cell types to facilitate the re-epithelialization of denuded areas [86]. In fact, signaling by TGF-β and the ECM are essential in promoting the activation and differentiation of myofibroblasts, which are the key cells involved in the repair of damaged epithelium and scar formation [86]. Working in concert with epithelial and endothelial cells, activated myofibroblasts secrete matrix metalloproteinases (MMPs) that digest injured tissues and facilitate the synthesis of a provisional ECM [87, 88]. Exposure of platelets and infiltrating immune cells to provisional ECM components elicits platelet degranulation, angiogenesis, and wound contraction, of which the latter response is mediated by myofibroblasts during the final stages of re-epithelialization [86, 87, 89]. Under normal circumstances, the inflammatory reactions within healed wounds resolve, thereby terminating type 2 EMT and stimulating the elimination of myofibroblasts via apoptosis [88]. However, sustained myofibroblast activation in conjunction with chronic inflammation underlies the initiation of fibrotic disorders due to unresolved type 2 EMT reactions [86, 87]. Myofibroblasts represent a specialized cell type that exhibit traits reminiscent of smooth muscle cells, particularly the expression of α-smooth muscle actin (α-SMA; [90, 91]). As a group, myofibroblasts derive from fibroblasts, from circulating progenitor cells, and from epithelial cells following their completion of EMT, which is typically assessed by monitoring the extent of α-SMA expression in fully transitioned cells [1]. Exacerbated α-SMA expression is also indicative of fibrotic states and fibroproliferative disorders [92–94], as well as correlates with increased tumor invasion and decreased survival rates in cancer patients [95]. Not surprisingly, α-SMA expression is induced by TGF-β via the concerted signaling inputs of Smad2/3, RhoA/Rock, and ERK1/2 [96–99]. Furthermore, integrin activation by laminin, fibronectin, and collagen also cooperates with TGF-β to induce EMT and myofibroblasts activation, an event coupled

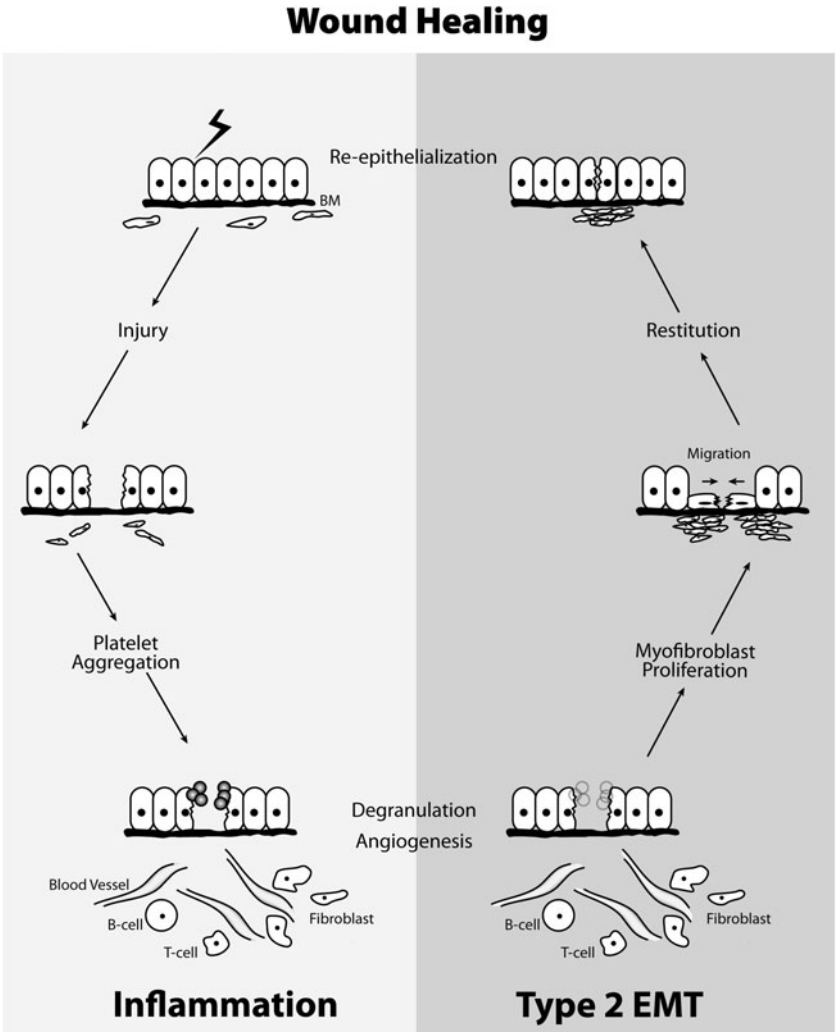

Figure 3 Type 2 EMT in wound healing and tissue regeneration. Normal epithelia that arose from type 1 EMT during development experience a noxious event or injury that triggers endothelial and epithelial cells to produce factors that mediate coagulation and clot formation. These events, coupled with enhanced MMP production facilitate the recruitment of immune cells and platelets to denuded wounds, leading to the production of a provisional ECM and activation of angiogenesis. TGF-β enhances the healing process by inducing EMT in myofibroblasts, which drives their differentiation, activation, and migration into denuded wounds to facilitate wound restitution, closure, and re-epithelialization. The role of TGF-β in regulating type 2 EMT during mammary gland fibrosis is discussed in the text.

to the formation of β-catenin:Smad2 signaling complexes [100–102]. Thus, in addition to its role in promoting type 2 EMT, aberrant TGF-β signaling also supports chronic inflammatory reactions that promote the establishment of fibroproliferative disorders in humans (Fig. 3).

At present, the overall importance of fibrotic reactions in promoting mammary tumorigenesis remains to be determined definitively. However, mammographically dense and fibrotic breast tissue have been linked to the increased incidence of mammary tumorigenesis [103, 104]. Along these lines, radiation therapy of breast cancers is associated with the development of fibrosis [105], and with the initiation of EMT via a TGF-β-dependent mechanism [106]. Moreover, mammary tumorigenesis is often accompanied by intense desmoplastic and fibrotic reactions, which elicit the formation of rigid tumor microenvironments that (*a*) enhance the selection and expansion of developing neoplasms, particularly that of late-stage metastatic tumors, and (*b*) predict for poor clinical outcomes in patients with breast cancer [107–109]. Interestingly, these aberrant cellular activities are highly reminiscent of those attributed to TGF-β [110], suggesting that TGF-β stimulation of EMT and fibrosis may promote the development and progression of mammary tumors. This supposition is bolstered by a recent study showing that inhibiting the cross-linking of collagen during mammary fibrosis reduces breast cancer progression in mice [111]. Collectively, these findings suggest that chemotherapeutic targeting of the EMT inducing activities of TGF-β may offer a novel two-pronged approach to alleviate breast cancer progression—namely, the inactivation of pathologic type 2 (i.e., fibrotic) and type 3 EMT (*see below*).

Mechanisms of Type 3 EMT Induced by TGF-β

The initiation of type 3 EMT is essential in facilitating cancer progression and metastasis, including that by mammary tumors [1, 2]. In addition, type 3 EMT is primarily distinguished from its type 1 and 2 counterparts by the cellular context in which this phenotypic transition transpires—namely, type 3 EMT occurs in oncogenically transformed cells that house a variety of genetic and epigenetic abnormalities that conspire with the molecular cascade that underlies EMT to elicit metastatic dissemination. Even more remarkably, TGF-β stimulation of EMT has been associated with the selection and expansion of breast cancer stem cells [16–19], which by their nature exhibit robust resistance to traditional cancer chemotherapies [112]. In the succeeding sections, we present our understanding of the molecular, cellular, and microenvironmental factors that contribute to the initiation of type 3 EMT by TGF-β in breast cancer cells (Fig. 4).

Canonical TGF-β Signaling During EMT

Although the activities attributed to Smad2 and Smad3 during TGF-β signaling are commonly conjoined in the scientific literature, recent findings indicate that these latent transcription factors do in fact mediate distinct biological activities in response to TGF-β [113]. These disparate functions mediated by Smads 2 and 3 also extend to EMT, which is inhibited by Smad2 and stimulated by Smad3. For instance, Smad2-deficiency enhances the development and progression of squamous cell carcinomas by elevating Snail expression and its induction of EMT [114], as well as elicits the acquisition of mesenchymal and fibrotic morphologies in hepatocytes [115]. Interestingly, oncogenic Ras cooperates with Smads 2 and 3 to drive the formation of spindle cells and their subsequent acquisition of EMT and metastatic phenotypes [116]. TGF-β stimulation of EMT in kidney cells results in the attenuated expression of SARA, an adapter molecule that facilitates the presentation of Smad2/3 to TβR-I [24]. Moreover, depleting cells of SARA increased the ubiquitination and degradation of Smad2, leading to the acquisition of EMT phenotypes [117]. In stark contrast, Smad3-deficiency (*a*) prevented TGF-β stimulation of EMT in lens [118] and renal [119] epithelial cells; (*b*) reduced the EMT and migratory abilities of keratinocytes to TGF-β [120, 121]; and (*c*) preserved an epithelial phenotype in hepatocytes stimulated by TGF-β [115]. Thus, these studies implicate a pro-EMT function associated with Smad3 activation, which is mirrored by the activation of Smad4 in pancreatic cells undergoing EMT induced by TGF-β [122]. Consistent with its designation as an inhibitory Smad, over expression of Smad7 is sufficient to abrogate the ability of TGF-β to induce EMT in epithelial cells of the breast [123], liver [124], and lens [125]. Collectively, these studies demonstrate that the expression and activities of TGF-β-regulated Smads are critical to the initiation and resolution of EMT in diverse epithelial cell lineages.

Smad2/3 activation also figures prominently in mediating TGF-β stimulation of EMT in MECs. Indeed, TGF-β signaling through Smads 2, 3, and 4 induce an EMT transcriptional program in normal MECs, a physiological reaction that is blocked by overexpression of Smad7 [123]. Interestingly, chronic TGF-β signaling elicits a metastable EMT phenotype in normal MECs that is characterized by reduced Smad2/3 signaling, leading to MEC resistance to the cytostatic and apoptotic activities of TGF-β [126]. Thus, EMT may underlie the conversion of TGF-β function from that of a tumor suppressor to a tumor promoter in mammary tumors (*see below*). Along these lines, Smad4-deficiency not only prevented TGF-β stimulation of EMT in normal and malignant MECs, but also alleviated its induction of bone metastasis by breast cancer cells in mice [127]. Furthermore, targeting and inactivating Smad2/3 signaling using aptamer

technology was observed to neutralize the ability of TGF-β to induce EMT in normal MECs [128]. Interestingly, Smad2 signaling has recently been shown to promote EMT in MECs by enhancing the DNA binding activity of the DNA methyltransferase, DNMT1, leading to chronic epigenetic silencing of epithelial-associated genes [129]. Finally, a recent study established that the aberrant coupling of TGF-β to BMP-regulated Smads (e.g., Smads 1 and 5) during mammary tumorigenesis confers a pro-migratory phenotype in breast cancer cells [130]. Although a role for this unusual coupling event in mediating EMT by TGF-β was not investigated, it is nonetheless tempting to speculate that inappropriate cross-talk between TGF-β superfamily members may contribute to the pathophysiological outcomes of EMT initiated by TGF-β. Future studies will need to address this question, as well as define the underlying relationship between Smad-dependent and -independent signaling during TGF-β stimulation of EMT in MECs (*see below*).

Noncanonical TGF-β Signaling During EMT

The aberrant amplification of noncanonical TGF-β signaling systems plays a salient role in mediating the ability of TGF-β to induce EMT in normal and malignant MECs, and in underlying the initiation of the "TGF-β Paradox." The function of noncanonical TGF-β effectors in coupling this cytokine to EMT and metastasis are discussed in the succeeding sections.

Rho-family GTPases

Oncogenic TGF-β signaling is often associated with the dysregulated activity of the Rho GTPase family, which includes RhoA/B/C, Rac1, and Cdc42 [1, 14, 20]. This family of small GTP-binding proteins are anchored to the plasma membrane where they regulate dynamic changes in cell adhesion, morphology, and motility in part by modulating the formation of filopodia (e.g., Cdc42), lamellipodia (e.g., Rac1), and actin stress fibers (e.g., RhoA) [131, 132]. For instance, RhoA activation mediates the dissolution of adhesion complexes at cell–cell junctions, while that of Rac1 actually promotes the formation of these same complexes [133, 134]. Additionally, constitutive activation of Cdc42 by TβR-III inhibits directional migration induced by TGF-β [135]. Interestingly, the expression and activation of RhoC enhances the invasion and EMT of breast [136], prostate [137], and colon [138] cancer cells. The ability of TGF-β to induce EMT in MECs requires the activation of RhoA and its downstream effector, $p160^{ROCK}$ [41]. In addition, the

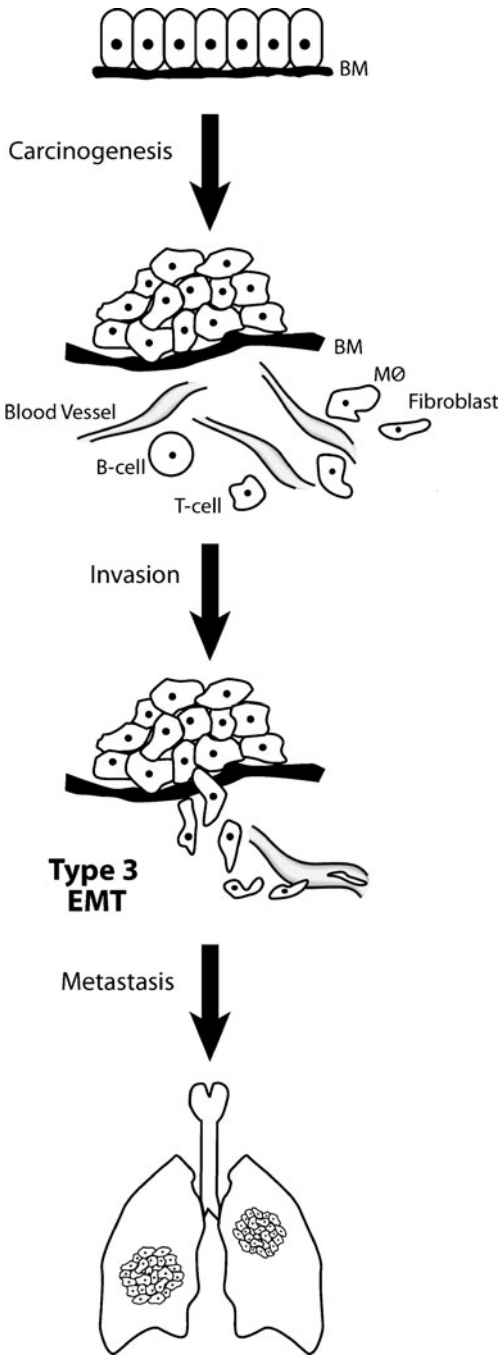

Figure 4 Type 3 EMT in cancer metastasis. Normal epithelia that arose from type I EMT during development experience a carcinogenic event that ultimately results in their oncogenic transformation and tumor formation. The development and progression of mammary tumors is accompanied by their acquisition of dysplastic and abnormal morphologies, and by their evolution in chronically inflamed tumor microenvironments, which further enhances their acquisition of EMT and fibrotic phenotypes. Through its ability to stimulate oncogenic EMT, TGF-β enables transitioned mammary carcinoma cells to invade the underlying basement membrane (BM) and escape the confines of the primary tumor. Once liberated, metastatic MECs undergo intravasation and traverse the blood stream prior to taking up residence at distant locales, an event that ultimately leads to disease recurrence and poor clinical outcomes in breast cancer patients. See text for specific details on the molecular mechanisms whereby TGF-β promotes type 3 EMT and metastasis in breast cancer cells.

coupling of TGF-β to RhoA and RhoC also correlates with altered expression of E-cadherin and α-SMA during EMT [96, 139]. Recently, TβR-II-mediated phosphorylation of Par6 was shown to underlie the ubiquitination and degradation of RhoA, leading to the dissolution of tight junctions during the acquisition of EMT and metastatic phenotypes in breast cancer cells [140, 141]. Along these lines, upregulation of miR-155 by canonical TGF-β signaling was observed to promote EMT in MECs by targeting the destruction of RhoA mRNA [142]. In stark contrast, reduced RhoA expression mediated by miR-31 was found to suppress, not promote, breast cancer metastasis [143, 144], suggesting an intricate and complex role for RhoA and its relatives in regulating the initiation of EMT and metastasis.

PI3K, AKT, and mTOR

Oncogenic TGF-β signaling and its stimulation of EMT also requires the activities of phosphoinositide 3-kinase (PI3K) and Akt, both of which confer proliferative and survival advantages to developing carcinomas [145]. In addition, PI3K and Akt also mediate TGF-β stimulation of EMT in MECs [40], an event that arises directly from TGF-β receptor signaling inputs, or indirectly through their transactivation of the receptors for EGF [146] and PDGF [147]. Interestingly, co-administration of TGF-β and EGF elicits an exaggerated EMT through the activation of ERK1/2 and PI3K/Akt [148]. Somewhat surprisingly, pharmacological inhibition of PI3K/Akt had no effect on the morphologic and phenotypic characteristics of EMT; however, this same treatment regimen did alleviate the ability of TGF-β and EGF to induce cell migration and invasion [148], suggesting that the morphologic and motile features of EMT may in fact be distinct physiological entities. Along these lines, inactivating mTOR pharmacologically with rapamycin prevents TGF-β from increasing the physical size of MECs, as well as from stimulating their migration and invasion [42]. As above, mTOR antagonism failed to impact the morphological features of EMT, which suggests that mTOR may facilitate the synergistic effects of TGF-β and EGF on EMT. Collectively, these studies highlight the essential function of the PI3K/Akt/mTOR pathway in promoting EMT and metastasis stimulated by TGF-β, while future studies need to clarify the underlying dissociation between "fibroblastoid-like" phenotypes and cell motility.

Nuclear Factor-κB

Nuclear factor-κB (NF-κB) plays a prominent role in regulating the initiation and resolution of inflammatory reactions, and in promoting the growth, angiogenesis, invasion, and survival of developing carcinomas [149]. In nontransformed tissues, TGF-β typically inhibits the activation of NF-κB [49, 50, 150], presumably by inducing the expression of IκBα [151], and by promoting the formation of TβR-III:β-arrestin2 complexes that prevent IκBα degradation [152]. In stark contrast, mammary tumorigenesis converts TGF-β from an inhibitor to a stimulator of NF-κB activity by inducing the formation of TβR-I:xIAP:TAB1:TAK1:IKKβ complexes [49, 50, 153]. Furthermore, activation of this noncanonical effector system by TGF-β was found to be essential for its induction of EMT in normal and malignant MECs [49, 50, 153, 154], and for its stimulation of mammary tumor development via activation of the innate immune system [49]. Moreover, the coupling of TGF-β to NF-κB activation underlies the ability for Ras-transformed breast cancer cells to colonize the lung [47, 155], and elicits the initiation of an autocrine Cox-2:PGE2:EP2 receptor signaling cascade that not only induces EMT in normal and malignant MECs, but also stimulates breast cancer metastasis [50, 51]. Finally, TGF-β stimulation of NF-κB in post-EMT cells stimulates their migration by establishing a SDF-1/CXCR4 signaling axis [156]. Collectively, these studies highlight the importance of NF-κB to the induction of EMT by TGF-β, while future studies need to assess the relative contribution of these events to enhanced survival and chemoresistance exhibited by post-EMT breast cancer cells.

MAP Kinases

The propensity of TGF-β to induce pathological EMT and metastasis is also associated with its stimulation of members of the MAP kinase family of protein kinases, including ERKs, JNKs, and p38 MAPKs [39, 157–159]. For instance, pharmacological inhibition of MEK1/2 prevents TGF-β from stimulating EMT and its characteristic formation of actin stress fibers and delocalization of E-cadherin and ZO-1 from the cell surface in MECs [158]. Similarly, methods that disrupt the coupling of TGF-β to JNK activation prevent the morphological and transcriptional changes associated with EMT [160, 161]. Along these lines, there exists a dynamic interplay between TGF-β and its production of various ECM components, which subsequently potentiate the activation of MAP kinase pathways during EMT. For instance, the expression of type I collagen activates PI3K, Akt, and JNK to induce EMT [162, 163]. Moreover, TGF-β in conjunction with vitronectin signaling is necessary in mediating TβR-II phosphorylation by Src, a phosphotransferase reaction operant in activating p38 MAPK and EMT in MECs [23, 45]. Likewise, the coupling of TGF-β to ERK1/2 and p38 MAPK activation is dependent upon the localization of TGF-β receptors to lipid rafts, not clathrin-coated pits, and as such, cholesterol-depleting methodologies are sufficient to block cell migration and EMT stimulated by TGF-β

[164]. Collectively, these studies implicate MAP kinases as crucial mediators linking the ability of TGF-β and ECM components to promote EMT.

Integrin-linked Kinase

The coupling of TGF-β to its noncanonical effectors during type 3 EMT is further exacerbated upon the activation of integrin-linked kinase (ILK), which is a Ser/Thr protein kinase coupled to the activation of small GTPases, PI3K/Akt, and MAP kinases [165–167]. ILK activation also leads to the inhibition of GSK-3β activity, which stabilizes β-catenin and facilitates its nuclear accumulation during EMT [166]. Indeed, elevated expression of ILK in MECs is associated with their decreased expression of E-cadherin and increased invasion [168], and with their oncogenic transformation by hyperactive ERK1/2 and Akt [169]. Along these lines, ILK-deficiency prevented TGF-β from stimulating cell migration and invasion in part by uncoupling this cytokine from regulation of the uPA/PAI-1 system [170]. Finally, TGF-β stimulation of Smad2/3 induces PINCH-1 expression, which interacts physically with ILK during the initiation of EMT and its consequential loss of E-cadherin and ZO-1 expression [171]. Collectively, these findings suggest that ILK interfaces integrin signaling with that of TGF-β, resulting in aberrant protease activation that drives the acquisition of EMT, invasive, and metastatic phenotypes.

Integrins and Focal Adhesion Signaling During EMT

Integrins and EMT Stimulated by TGF-β

Communication within cell microenvironments is controlled in part by integrins, which govern cell adhesion, migration, and invasion, as well as cell proliferation and survival [172, 173]. Cells undergoing neoplastic transformation exhibit dramatically altered integrin expression profiles, as well as altered integrin affinities for ECM substrates, both of which enhance cancer cell invasion and metastasis [174]. As a receptor family, integrins are unique in their capacity to physically link the ECM to cytoskeletal system within cells, thus enabling the efficient propagation of mechanotransduction in a bidirectional manner [111, 175]. In addition, focal adhesion kinase (FAK) serves as a molecular bridge that links integrins to the receptors to EGF and PDGF, thereby conferring cell migration activities to these growth factors [176, 177]. Integrins also play an important role in eliciting EMT and cell migration stimulated by TGF-β. For instance, αvβ6 and αvβ8 integrins bind latent TGF-β complexes and elicit the presentation of TGF-β to its cell surface receptors [178], presumably by promoting matrix metalloproteinase (MMP)-14 activation [27, 179]. Along these lines, TGF-β induces the expression of α3β1 and αvβ3 integrins, which confer migration and invasion properties to MECs [45, 46, 56, 157]. Administering neutralizing β1 integrin antibodies to MECs uncoupled TGF-β from the activation of p38 MAPK and the induction of EMT [157]. In addition, genetic or pharmacologic inactivation of αvβ3 integrin in normal and malignant MECs prevented TGF-β from inducing EMT and pulmonary metastasis [45, 46, 56]. Mechanistically, upregulated β3 integrin expression stimulated by TGF-β results in the FAK-dependent formation of β3 integrin:TβR-II complexes that promote the activation of Src and its phosphorylation of TβR-II at Y284 [56, 180]. Upon its phosphorylation, Y284 coordinates the recruitment and binding of the SH2-domain proteins, Grb2 and ShcA, which promote p38 MAPK activation and the initiation of EMT [45, 46, 56]. Importantly, measures that disrupt this oncogenic TGF-β signaling axis completely abrogate the ability of TGF-β to induce EMT, and to promote the metastasis of breast cancer cells to the lung [46, 180, 181] and bone [182]. Recently, we observed an interesting interplay between β1 and β3 integrins in regulating MEC response to TGF-β, such that genetic inactivation of β1 integrin in MECs elicits a compensatory upregulation of β3 integrin expression that impacts the coupling of TGF-β to EMT and cell motility (J.G. Parvani and W.P. Schiemann, *unpublished observation*). These findings implicate "integrin switching" as a potentially dangerous mechanism that may enable metastatic breast cancers to escape integrin-based chemotherapies. Future studies clearly need to investigate the validity of this hypothesis, as well as to identify the repertoire of integrins capable of regulating the diverse pathophysiological activities of TGF-β in normal and malignant MECs.

Nonreceptor Protein Tyrosine Kinases and Adapter Molecules of Focal Adhesion Complexes

As mentioned above, EMT and oncogenic TGF-β signaling transpires through a β3 integrin:FAK:Src:phospho-Y284-TβR-II:Grb2:p38 MAPK signaling axis that forms constitutively in basal-like breast cancer cells [45, 46, 56, 180, 182]. The importance of this αvβ3 integrin-based signaling axis in promoting the oncogenic activities of TGF-β in other genetically distinct breast cancer subtypes remains unexplored; however, a number of recent studies have identified essential functions for a variety of focal adhesion complex proteins in mediating the coupling of TGF-β to EMT and metastasis. Indeed, in addition to its ability to coordinate the formation of β3 integrin:TβR-II complexes, FAK expression and activity are essential in coupling TGF-β to EMT and p38 MAPK activation, and to inducing pulmonary metastasis of breast cancer cells [180]. Adjuvant FAK chemotherapy also inhibited breast cancer growth by suppressing the ability of macrophages to infiltrate the

primary mammary tumor [180], suggesting that the tumor promoting activities of FAK manifest in carcinoma cells and their associated stromal compartment. Along these lines, FAK is essential in mediating TGF-β stimulation of E-cadherin redistribution and α-SMA expression during EMT [183, 184]. In addition, the phosphorylation and activation of p130Cas functions as a molecular rheostat that governs the balance between canonical and noncanonical TGF-β signaling inputs. Indeed, activated p130Cas forms a heteromeric complex with Smad3 and TβR-I, which diminishes the phosphorylation of Smad3 and uncouples TGF-β from regulation of cell cycle progression [185]. Interestingly, breast cancer patients whose tumors express abnormally high levels of p130Cas exhibit tamoxifen- and adriamycin-resistance, as well as diminished time to disease recurrence [186, 187]. Likewise, elevated p130Cas expression significantly reduced the latency of mammary tumor formation driven by transgenic expression of Her2/Neu [188]. We recently observed elevated p130Cas expression to mark the development of metastasis in breast cancers, and to skew the balance of TGF-β signaling from canonical to noncanonical effectors in metastatic MECs [189].

Similar to p130Cas, the adapter molecules Hic5 and Dab2 also promote the coupling of TGF-β to its noncanonical effectors during EMT. Indeed, Hic5 is a member of the paxillin superfamily that functions in the cytoplasm as a component of focal adhesion complexes [190], and in the nucleus as a transcriptional co-activator of the androgen receptor [191]. Polarized MECs express low levels of Hic5, which are increased rapidly during EMT via a RhoA/ROCK-dependent pathway [190, 192]. Interestingly, the LIM domain of Hic5 binds and inactivates Smad3 and Smad7 in prostate cancer cells [193, 194], which collectively diminishes Smad-dependent gene expression (i.e., targeting Smad3) in the context of enhanced TGF-β receptor signaling (i.e., targeting Smad7). Whether Hic-5 possesses similar anti-Smad activity in breast cancers remains unknown; however, it is tempting to speculate that Hic5 may cooperate with p130Cas in amplifying the coupling of TGF-β to its noncanonical effectors.

Finally, the adaptor molecule, Dab2 (Disabled-2) regulates the dynamics associated with the remodeling of the actin cytoskeleton during MEC adhesion and migration [195]. In contrast to p130Cas and Hic5, Dab2 associates with TGF-β receptors and facilitates their activation of Smad2/3 [26], as well as that of TAK1 and JNK, which stimulate fibronectin expression and cell migration during EMT [160, 196]. In addition, TGF-β stimulation of EMT assembles Dab2:β1 integrin complexes that induce FAK activation. Mechanistically, translation of Dab2 mRNA is strongly repressed in polarized MECs by the actions of hnRNP E1, which binds structural elements in the 3′UTR of Dab2 transcripts. When activated by TGF-β, Akt2 phosphorylates and releases hnRNP E1 from Dab2 mRNA, thereby enabling the production of Dab2 and its initiation of EMT in MECs [197]. Future studies need to identify other genes targeted by this novel post-transcriptional regulon, as well as to define their role in mediating the pathophysiological outcomes of EMT stimulated by TGF-β.

Gene Regulation-Coupled to EMT Induced by TGF-β

The ultimate phenotypic change associated with the activation of canonical and noncanonical TGF-β signaling inputs derives from altered patterns of gene expression and repression that transpire in a cell- and context-specific manner. In the succeeding sections, we highlight the important transcriptional mediators operant in driving and fine-tuning the EMT transcriptome targeted by TGF-β in normal and malignant MECs.

Nuclear Transcription Factors Targeted by TGF-β During EMT

Members of the Snail (SNAI1 and SNAI2/Slug), ZEB (ZEB1 and ZEB2/SIP1), basic helix-loop-helix (Twist), and Six family of homeobox (Six1) transcription factors are considered to be master regulators of EMT, including that stimulated by TGF-β [198, 199]. As a group, these transcription factors play essential roles in mediating type I EMT during embryogenesis and tissue morphogenesis; however, their inappropriate reactivation of developmental EMT programs during tumorigenesis is considered a hallmark of disease progression and metastasis initiation [61]. Indeed, activated Snail molecules readily form complexes with Smads 3 and 4 that collectively target conserved E-box sequences in the promoters for E-cadherin, occludin, and claudin, which strongly represses their expression and inactivates adherens (i.e., E-cadherin) and tight junction (i.e., occludins and claudin) complexes during EMT [200, 201]. Similar targeting and inactivation of E-cadherin is associated with all of the aforementioned transcription factors, whose underlying roles in mediating the pathophysiology of EMT has been the subject of several recent reviews (*see* [198, 199]). Interestingly, dysregulated Myc expression has been observed to function cooperatively with Smad4 to induce an EMT-related transcriptional profile in normal and malignant MECs [202]. Likewise, the reactivation of fibulin-5 expression in transitioning MECs initiated a positive-feedback loop that sensitized MECs to the EMT-promoting activities of TGF-β, an event dependent upon the synergistic induction of Twist expression by fibulin-5 and TGF-β [203]. In addition, TGF-β stimulation of Smad3 results in upregulated Mdm2 expression, which destabilizes p53 during the initiation of breast cancer EMT and metastasis [204]. Smad3/4 signaling

also promotes the expression of HMGA2, which stimulates the EMT transcriptional program by inducing the expression of Snail, Slug, and Twist, while simultaneously repressing that of Id2 [205]. Finally, members of the homeobox family of transcription factors have also been implicated in mediating EMT induced by TGF-β. For instance, inappropriate LBX1 (**l**ady**b**ird homeobo**x 1**) expression drives EMT and its expansion of breast cancer stem cells by stimulating the expression of TGF-β2, Snail, and ZEBs 1 and 2 [206]. Similarly, aberrant reactivation of the homeoprotein Six1 promotes the acquisition of EMT and metastatic phenotypes in breast cancer cells in part by upregulating the messages transduced through the TGF-β signaling system [85]. Collectively, these findings highlight the role of developmental EMT pathways that enhance the oncogenic activities of TGF-β during mammary tumorigenesis.

Estrogen Receptor-α

Estrogen receptor (ER) status has long been recognized as an important prognostic marker in developing breast cancers, particularly in terms of its diagnostic and therapeutic value. Indeed, the loss of ER-α expression during mammary tumorigenesis is associated with poor clinical outcomes, and with increased likelihood of breast cancer metastasis and disease recurrence [207]. More recently, ER-α has been linked to the initiation of EMT through its ability to activate metastasis associated protein 3 (MTA3) in MECs [208, 209]. Mechanistically, MTA3 serves as a subunit of the Mi-2/NuRD chromatin remodeling complex, which represses the expression of Snail [208]. Thus, mammary tumors that have lost expression of ER-α exhibit reduced MTA3 activity that results in the inappropriate expression of Snail and its subsequent stimulation of EMT [208, 209]. This process is further enhanced by the ability of Snail to repress ER-α expression, and to induce the expression of components of the TGF-β signaling system (e.g., TGF-β2 and TβR-II; [208]), thereby creating a powerful positive feedback loop to further potentiate the acquisition and stabilization of EMT phenotypes. Moreover, ER-α interacts physically with Smad3 and inhibits its ability to regulate gene expression [210]. Thus, the loss of ER-α expression and activity may play a prominent role in engendering the initiation of oncogenic TGF-β signaling in normal and malignant MECs [8]. In fact, aberrant cytoplasmic localization of ER-α has recently been proposed as a novel histopathological marker to identify sarcomatoid breast cancers in vivo [211]. Future studies clearly need to elucidate molecular mechanisms that underlie the dynamic relationship between ER-α and TGF-β in regulating EMT, and to identify novel biomarkers capable of staging and stratifying breast cancer patients on the basis of their EMT, ER-α, and TGF-β status.

TGF-β and microRNAs

Recent studies have implicated the aberrant expression of numerous microRNAs (miRs) in the initiation of EMT, and in the development and progression of mammary tumors [212–214]. Indeed, members of the miR-200 family of microRNAs maintain polarized epithelial phenotypes by repressing the expression of the EMT-inducing transcription factors ZEB1 and ZEB2/SIP-1. Accordingly, monitoring the expression of miR-200 family members can be used to distinguish well-differentiated and immotile tumors that express E-cadherin from their poorly-differentiated and highly motile counterparts that express vimentin [215]. In addition, TGF-β abrogates the expression of miR-200 family members, leading to the expression of ZEB1 and ZEB2 and their consequential downregulation of E-cadherin expression to initiate EMT [216, 217]. Not surprisingly, miR-200 family member expression is frequently downregulated in invasive and metastatic mammary tumors, particularly those possessing mesenchymal-type breast cancer cells [216]. Once expressed, ZEB1 can further repress the expression of miR-200 family members, thereby stabilizing the EMT phenotype in transitioning MECs [218].

The stimulation of EMT by TGF-β also transpires through its regulation of additional miRs. Indeed, TGF-β stimulation of normal MECs elicits their upregulated expression of miR-155 via a Smad4-dependent pathway. Once expressed, miR-155 participates in EMT by downregulating RhoA expression, leading to the dissolution of tight junctions [142]. Along these lines, upregulated expression of miR-21 induced by TGF-β abrogates the expression of several tumor suppressors, including *a*) peroxisome proliferator-activated receptor; *b*) tissue inhibitor of metalloproteinase-3; *c*) programmed cell death 4; and *d*) AT-rich interactive domain 1A [219]. Additionally, miR-21 expression also participates in the initiation of EMT by downregulating the expression of tropomyosin, leading to enhanced breast cancer motility, invasion, and anchorage-independent growth [220, 221]. Thus, the coupling of TGF-β to the regulation of miR expression and activity affords new avenues to potentially manipulate the pathophysiology associated with type 3 EMT in mammary tumors. As such, future studies need to rapidly and accurately define the cellular targets of EMT-associated miRs, and to determine the molecular mechanisms operant in regulating their expression in transitioning MECs.

DNA Hypermethylation

Hypermethylation and silencing of the E-cadherin promoter has been linked to the initiation of EMT, migration, and invasion in breast cancer cells [222–224]. More recently, TGF-β signaling was observed to maintain DNA methylation patterns during EMT, resulting in the silencing of E-

cadherin (*CDH1*), the tight junction genes *CGN* and *CLDN4*, and the protease *KLK10/NES1*. Mechanistically, overexpressing Smad7 in MECs or rendering them deficient in Smad2 inhibited the activity of the DNA methyltransferase, DNMT1, which suppressed EMT and cell motility by restoring E-cadherin expression [129]. Along these lines, miR-200c and its relative, miR-141, normally inhibit the initiation of EMT and metastasis in MECs by suppressing the expression of ZEB1/2 [216, 217]. However, during mammary tumorigenesis, aberrant DNA methylation inactivates expression of these miR-200 family members, leading to the acquisition of EMT and metastatic phenotypes in developing and progressing mammary tumors [225]. Moreover, E-cadherin-deficiency that arises during EMT may in fact function as an initiating signal coupled to the expanded and directed hypermethylation of genes normally operant in suppressing mammary tumorigenesis [226]. For instance, hypermethylation of the E-cadherin promoter marks Ras-transformed MECs that have undergone a stable EMT induced by serum versus a transient EMT induced by TGF-β [226]. Collectively, these studies establish an essential role for DNA methylation in facilitating type 3 EMT stimulated by TGF-β, and in differentially stabilizing the EMT phenotype in response to varying stimuli.

Microenvironmental Inputs During EMT Stimulated by TGF-β

Tissue homeostasis in the breast is maintained by the balanced integration of signaling inputs derived from various tissue and cell architectures, and from their supporting microenvironment and ECM. Indeed, whereas normal mammary tissue specification requires reciprocal signaling inputs from distinct cell types and matrix proteins, the phenotype of developing mammary carcinomas is similarly dictated by the dynamic interplay between malignant cells and their accompanying stroma, which houses fibroblasts, endothelial cells, and a variety of infiltrating immune and progenitor cells [227–229]. Along these lines, desmoplasia and fibrosis during mammary tumorigenesis can drive disease progression in a manner that mimics the oncogenic activities of TGF-β [110, 230], suggesting that the interactions between MECs and their supporting stromal constituents play pivotal roles in regulating EMT and metastasis stimulated by TGF-β. Recent findings pertaining to the connections between the microenvironment and EMT are summarized in the following sections.

Adherens Junctions and EMT

A hallmark of EMT is the dissolution of cell–cell junctions, particularly adherens junctions which derive from the homotypic interactions between adjacent E-cadherin molecules housed on neighboring MECs. Similar to integrins, the cytoplasmic domain of E-cadherin is tethered to the cytoskeleton through a heteromeric complex consisting of the α-, β-, and γ-catenins, which collectively serve in marking differentiated MECs and suppressing their tumorigenesis [231, 232]. Interestingly, the process of EMT is often characterized by "cadherin switching," a term referring to the ability of E-cadherin expression and activity to give way to that of N-cadherin as MECs acquire mesenchymal phenotypes [233]. Functional inactivation of E-cadherin transpires through a variety of mechanisms, including hypermethylation and epigenetic silencing of its promoter [234], as well as protease-mediated cleavage and shedding of its ectodomain [231]. In rare cases, mutational inactivation of the E-cadherin gene, *CDH1*, has been observed and linked to increased risk for cancer development in affected individuals [235]. However, transcriptional repression is by far the most common mechanism employed by transitioning MECs to downregulate their expression of E-cadherin. Indeed, TGF-β stimulation of EMT represses E-cadherin expression primarily by targeting the expression of Snail, ZEB, and bHLH family members (*see above*). Moreover, upregulated expression of the mesenchymal cadherins, N-cadherin and cadherin-11, occur concomitantly with the loss of E-cadherin expression and correlate with increased tumor invasiveness and poor clinical outcomes [236–239]. At present, the dynamic relationship between E- and N-cadherin in mediating TGF-β stimulation of EMT and metastasis remains unresolved, as does the manner through which bifurcated TGF-β signals coupled selectively to epithelial versus mesenchymal transcriptional programs influence the pathophysiological outcomes of EMT induced by TGF-β [50, 180].

Neuronal Cell Adhesion Molecule and EMT

Neuronal cell adhesion molecule (NCAM) belongs to the immunoglobulin superfamily and mediates calcium-independent cell–cell adhesion [240]. Inappropriate NCAM expression has been associated with cancer progression and poor prognosis in cancers of the pancreas and colorectal system [233, 240]. In addition, TGF-β stimulation of EMT in MECs significantly upregulates their expression of NCAM, a reaction that requires the activation of Smad4 and inactivation of E-cadherin. Mechanistically, NCAM translocates to lipid rafts and activates a $p59^{fyn}$:FAK:β1 integrin signaling axis that promotes EMT and cell invasion [241]. Interestingly, EMT induced by TGF-β also activates matrix metalloproteinase (MMP)-28, which cleaves NCAM and latent TGF-β complexes [242]. Thus, it remains unclear how upregulated NCAM expression and cleavage ultimately impact the ability of TGF-β to stimulate EMT in normal and malignant cells.

Protease Activation During EMT Induced by TGF-β

Matrix Metalloproteinases

One of the hallmarks of EMT is its propensity to bestow motile phenotypes in previously immotile cells [1–3]. Matrix metalloproteinases (MMPs) are a superfamily of transmembrane and secreted endopeptidases that function in degrading a variety of ECM components, cytokines, and cell surface proteins and receptors. The net effect of these various MMP activities results in dramatic effects on cell differentiation, invasion, and EMT [243]. Members of the MMP superfamily (e.g., MMPs 2, 9, 13, and 14; [179, 244–246]) also function in mediating the cleavage of latent TGF-β complexes, which releases mature TGF-β and initiates transmembrane signaling in neighboring MECs, as well as mediates E-cadherin cleavage and breast cancer progression [243, 247, 248]. Along these lines, TGF-β is a potent regulator of the expression of MMPs 2, 9, and 13 [60, 203, 249–252], thereby establishing a positive autocrine TGF-β signaling loop that (*a*) drives breast cancer EMT, invasion, and metastasis, and (*b*) is readily suppressed by constitutive c-Abl activation in normal and malignant MECs [60]. Future studies need to address how aberrant MMP activation contributes to the initiation of type 2 EMT (*see above*), and how these events ultimately impact the initiation of type 3 EMT and metastasis by TGF-β in mammary tumors.

Urokinase Plasminogen Activator and Plasminogen Activator Inhibitor-1

Urokinase plasminogen activator (uPA) is serine protease that plays important roles in regulating the migration and invasion of breast cancer cells in part via its conversion of inactive plasminogen into active plasmin. Elevated expression of uPA correlates with increased tumor aggressiveness and poor clinical outcomes for a variety of cancers, including those of the breast [253–255]. The role of uPA in promoting breast cancer progression and metastasis has been recapitulated in a mouse model of mammary tumorigenesis [255], and in a chick chorioallantoic membrane model of breast cancer metastasis, which also associated upregulated uPA expression with hypoxia-induced EMT [256]. TGF-β induces uPA expression by activating JNK- and ILK-dependent signaling systems that functionally converge to induce EMT and increased cell motility [161, 170]. As noted previously, these findings point to a prominent role of noncanonical TGF-β effectors in mediating the stimulation of EMT by TGF-β. Accordingly, the expression and activity of FAK is essential in stimulating the production of uPA and its initiation of metastasis in 4T1 cells [255], which we [46, 49, 51, 60, 180, 189, 203] and others [257–259] established as a late-stage model of TGF-β-responsive breast cancer. In addition, hypoxia-induced EMT stimulates the expression of the uPA receptor, uPAR, which interacts physically with α3β1 integrin and promotes the activation of Src, Akt, Rac1, and GSK-3β. The end product of these signaling inputs elicits Snail and Slug expression, which drive the acquisition of EMT phenotypes and loss of E-cadherin in transitioning cells [256, 260]. Thus, future studies clearly need to define the connections linking the activation of these noncanonical TGF-β effectors to the formation of uPAR:integrin complexes.

Inappropriate activation of uPA is held in check by the expression of plasminogen activator inhibitor (PAI)-1 and PAI-2, which bind uPA:uPAR complexes and induce their internalization and degradation [253]. Thus, elevated PAI-1/2 expression would be predicted to alleviate the EMT and metastasis promoting properties of uPA, an assumption that has been validated in a panel of breast, ovarian, endometrial, cervical, and osteosarcoma cell lines [261]. Quite surprisingly, PAI-1 polymorphisms or its elevated expression has also been linked to enhanced disease progression and metastasis development, and to decreased survival in breast cancer patients [253, 261, 262]. TGF-β is a master regulator of PAI-1 expression, doing so through its stimulation of canonical and noncanonical effector systems (*see* [263]). In addition to binding and inactivating uPA:uPAR complexes, PAI-1 also interacts with vitronectin and prevents its activation of integrins [261], an event that may influence the coupling of integrin signaling to MEC migration. Thus, future studies need to clarify the tumor suppressing and promoting activities of PAI-1, particularly with respect to its role in mediating EMT and oncogenic TGF-β signaling in normal and malignant MECs.

Collagen

TGF-β has long be recognized for its ability to induce the expression of collagens [264], which function as important structural components of the ECM and serve as prominent ligands for integrins [243]. In addition, activation of p38 MAPK by TGF-β upregulates the expression of MMPs 2 and 9, which cleave collagen to produce biologically active fragments that readily promote MEC migration and invasion [252]. Along these lines, TGF-β stimulates breast cancer cells to upregulate their expression of the collagen receptor Endo180, which internalizes collagen and induces the growth of mammary tumors in mice [265]. More recently, collagen binding to β1 integrins has been shown to activate TGF-β receptor signaling independent of TGF-β ligands, leading to the activation of FAK and Src that culminate in the stimulation of Smad2/3 activity in MECs [266]. Collectively, these findings establish that TGF-β and collagen engage one another in a reciprocal relationship, yet how these events impact the ability of TGF-β to promote the

acquisition of EMT and metastatic phenotypes mammary tumors remains an unresolved and interesting question.

Fibronectin

Fibronectin is an important component of the ECM and its expression is upregulated dramatically by TGF-β during EMT [264, 267]. Functionally, fibronectin acts as a ligand for integrins during cell adhesion and migration, particularly in Ras-transformed MECs which concomitantly upregulate α5β1 integrin [268]. Importantly, administration of neutralizing antibodies against α5 integrin blocked the ability of fibronectin and TGF-β to stimulate EMT and cell motility in MECs [268]. In addition, fibronectin expression has been shown to modulate the response of cells to TGF-β. For instance, the ability of TGF-β to induce anchorage-independent growth in fibroblasts could be recapitulated by administration of fibronectin, whose activation of cell signaling was dependent upon integrin ligation [264]. Besides its ability to enhance TGF-β stimulation of EMT in bronchial epithelial cells [269], fibronectin expression has also been linked to the development of the "premetastatic niche," which serves as a depot to recruit circulating progenitor cells and metastatic carcinoma cells to sites of secondary tumor growth [270, 271]. Future studies need to assess the relative contributions of TGF-β and fibronectin in mediating EMT and its potential involvement regulating the formation of metastatic niches during breast cancer progression.

Conclusions and Future Directions

TGF-β is universally recognized as a master regulator of EMT, including that occurring during embryonic development and tissue morphogenesis (i.e., type 1 EMT), during wound healing and tissue fibrosis (i.e., type 2 EM), and during invasion and metastasis (i.e., type 3 EMT). Equally exciting are recent findings linking EMT stimulated by TGF-β to the acquisition of "*stem-like*" phenotypes in developing and progressing mammary tumors [16–19]. Thus, pharmacological targeting of the TGF-β signaling system to alleviate EMT may elicit chemosensitivity in cancer stem cells previously resistant to standard treatment regimens, a supposition supported by recent findings obtained in a preclinical model of breast cancer progression [19]. A corollary states that the phenomenon underlying selection and expansion of cancer stem cells via EMT may be "*druggable*" in clinical settings. Accordingly, high-throughput chemical screening technologies identified salinomycin as a novel agent capable of targeting breast cancer stem cells, thereby inhibiting mammary tumor growth in part by promoting epithelial differentiation [272]. Future studies need to determine the efficacy of salinomycin and related compounds in antagonizing EMT stimulated by TGF-β in normal and malignant MECs, as well as investigate the relative contribution of cell microenvironments in mediating the various pathophysiological outcomes of EMT induced by TGF-β. Ultimately, these findings will form the foundation necessary to manipulate EMT and its initiation of the "TGF-β Paradox" in mammary tumors, and as such, to one day improve the clinical course of patients with metastatic breast cancer.

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ORIGINAL ARTICLE

Transforming growth factor-β-induced epithelial–mesenchymal transition facilitates epidermal growth factor-dependent breast cancer progression

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Transforming growth factor-β (TGF-β) and epidermal growth factor (EGF) have critical roles in regulating the metastasis of aggressive breast cancers, yet the impact of epithelial–mesenchymal transition (EMT) induced by TGF-β in altering the response of breast cancer cells to EGF remains unknown. We show in this study that murine metastatic 4T1 breast cancer cells formed compact and dense spheroids when cultured under three-dimensional (3D) conditions, which was in sharp contrast to the branching phenotypes exhibited by their nonmetastatic counterparts. Using the human MCF10A series, we show that epithelial-type and nonmetastatic breast cancer cells were unable to invade to EGF, whereas their mesenchymal-type and metastatic counterparts readily invaded to EGF. Furthermore, EMT induced by TGF-β was sufficient to manifest dense spheroid morphologies, a phenotype that increased primary tumor exit and invasion to EGF. Post-EMT invasion to EGF was dependent on increased activation of EGF receptor (EGFR) and p38 mitogen-activated protein kinase, all of which could be abrogated either by pharmacologic (PF-562271) or by genetic (shRNA) targeting of focal adhesion kinase (FAK). Mechanistically, EMT induced by TGF-β increased cell-surface levels of EGFR and prevented its physical interaction with E-cadherin, leading instead to the formation of oncogenic signaling complexes with TβR-II. Elevated EGFR expression was sufficient to transform normal mammary epithelial cells, and to progress their 3D morphology from that of hollow acini to branched structures characteristic of nonmetastatic breast cancer cells. Importantly, we show that TGF-β stimulation of EMT enabled this EGFR-driven breast cancer model to abandon their inherent branching architecture and form large, undifferentiated masses that were hyperinvasive to EGF and showed increased pulmonary tumor growth upon tail vein injection. Finally, chemotherapeutic targeting of FAK was sufficient to revert the aggressive behaviors of these structures. Collectively, this investigation has identified a novel EMT-based approach to neutralize the oncogenic activities of EGF and TGF-β in aggressive and invasive forms of breast cancer.

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Introduction

The process of epithelial–mesenchymal transition (EMT) induced by transforming growth factor-β (TGF-β) is well established as a critical mechanism of tumor progression (Zavadil and Bottinger, 2005; Moustakas and Heldin, 2007; Kalluri and Weinberg, 2009; Wendt *et al*., 2009a); however, how these TGF-β-dependent events impact the response to growth factors during metastasis remains incompletely understood (Cowin and Welch, 2007; Moustakas and Heldin, 2007; Wendt *et al*., 2009a). Equally mysterious are the reasons underlying the failure of science and medicine to readily detect the classical mesenchymal and sarcomatoid phenotypes exhibited by fully transitioned carcinoma cells at sites of secondary metastases, which in theory should be enriched in these dedifferentiated and post-EMT cell types (Tarin *et al*., 2005). It therefore stands to reason that deciphering the molecular mechanisms that underlie the interplay between EMT and its counterpart mesenchymal–epithelial transition (Hugo *et al*., 2007) may offer new inroads into targeting tumor metastasis.

Applying genomic analyses to human breast cancers has resulted in the identification and classification of at least five genetically distinct breast cancer subtypes (Perou *et al*., 2000; Sorlie *et al*., 2001, 2003), of which the basal-like 'triple-negative' subtype remains the most intractable to clinical intervention. More recently, elevated expression of ErbB1/epidermal growth factor (EGF) receptor (EGFR) in basal-like tumors has been identified as a highly predictive marker for poor clinical outcomes (Tischkowitz *et al*., 2007). Indeed, synergistic activity between TGF-β and EGF in stimulating EMT has been identified (Saha *et al*., 1999), whereas the actual process of EMT has been associated with the development of chemoresistance to EGFR-targeted therapies in carcinoma cells (Thomson *et al*., 2005, 2008; Buck *et al*., 2007; Barr *et al*., 2008). Because TGF-β is a master

regulator of EMT, we hypothesized that EMT stimulated by TGF-β would induce a fundamental change in how tumor cells sense and respond to their surrounding microenvironment, particularly to EGF (Joo *et al*., 2007; Wang *et al*., 2009). Along these lines, recent studies suggest that aberrant EGFR signaling reflects the inactivation of E-cadherin, a hallmark of EMT (Miettinen *et al*., 1994; Takahashi and Suzuki, 1996; Wilding *et al*., 1996; Bremm *et al*., 2008). However, other studies implicate a novel paracrine signaling loop that transpires between carcinoma cells and tumor-infiltrating macrophages that comprise the actions of TGF-β, EGF and colony-stimulating factor-1 in promoting breast cancer cell migration and invasion to EGF (Wyckoff *et al*., 2000, 2004; DeNardo *et al*., 2009).

Focal adhesion kinase (FAK) is a multifunctional protein tyrosine kinase and scaffolding molecule that links transmembrane signaling inputs arising from integrins and growth factor receptors to intracellular effectors (Mitra *et al*., 2006). Along these lines, FAK has been shown to interact directly with the intracellular domain of EGFR to facilitate its downstream signaling and activation of cell motility (Sieg *et al*., 2000). Recently, analyses performed by our laboratory established a key role for FAK in physically associating integrins with TβR-II (Wendt and Schiemann, 2009). Moreover, we showed that the chemotherapeutic targeting of FAK prevented the infiltration of macrophages into primary mammary tumors (Wendt and Schiemann, 2009). Clearly, these and other studies have established FAK as a key factor in mediating EMT and metastasis stimulated by TGF-β (Cicchini *et al*., 2008; Ding *et al*., 2008); however, whether FAK facilitates the potential pathophysiological activities between TGF-β and EGF remains unknown. Thus, the objectives of this study were to (1) determine how the response of mammary epithelial cells (MECs) to EGF was altered by EMT induced by TGF-β; (2) establish the signaling mechanisms responsible for eliciting the aberrant responses of post-EMT MECs to EGF and (3) characterize the three-dimensional (3D) morphology of resulting hyperinvasive, post-EMT MECs.

Results

Metastatic breast cancer cells grow as dense cell structures manifested by TGF-β-induced EMT

Recent studies strongly suggest that the morphology and cell signaling responses of mammary tumors are more accurately recapitulated *in vitro* using 3D-organotypic systems as compared with growing cells on plastic (Park *et al*. 2000; Kenny *et al*., 2007; Wendt *et al*., 2009b). To this end, we used a well-characterized murine mammary carcinoma progression series comprised of several isogenic cell lines that possess varying metastatic proficiencies and include: (1) noninvasive and nonmetastatic 67NR cells, which form primary tumors that cannot enter the circulation; (2) invasive and nonmetastatic 4T07 cells, which traverse the circulation and fail to establish secondary tumors in the lung and (3) highly metastatic 4T1 cells, which disseminate widely and colonize multiple organ sites (Aslakson and Miller, 1992). Surprisingly, both nonmetastatic cell lines formed highly branched 3D structures, whereas metastatic 4T1 cells formed dense spheroids to lobular-like structures that were devoid of branching (Figure 1a). To verify the metastatic designation of these MEC derivatives, we engineered them to stably express luciferase and then engrafted them onto the mammary fat pad, and pulmonary metastasis was tracked over time using bioluminescent imaging. As expected, only fully metastatic 4T1 cells readily formed secondary metastatic lesions when engrafted onto the mammary fat pads of mice (Figure 1b). Given the recent studies from our lab and others identifying a critical role of EMT in driving breast cancer progression, we sought to examine how EMT induced by TGF-β affected subsequent 3D culture morphologies and tumor metastasis (Padua *et al*., 2008; Wendt and Schiemann, 2009; Wendt *et al*., 2009a). Interestingly, following a prolonged treatment with TGF-β, 4T07 cells showed a 3D morphology that was highly reminiscent of the metastatic 4T1 cells (Figures 1c and a). To verify their EMT status, lysates of TGF-β-treated 4T07 cells were analyzed for decreased expression of E-cadherin and increased expression of vimentin (Figure 1d). Given the somewhat counterintuitive nature of this TGF-β-induced 3D EMT morphology, we used the 67NR cell line that grew as a mixture of independent spheroid and branched structures (Supplementary Figure 1). Indeed, by physically isolating the spheroid structures from 3D cultures, expanding them on a plastic growth surface and then placing them back into 3D cultures we definitively showed that spheroid structures in 3D cultures directly correspond to a classic mesenchymal morphology when cultured plastic (Supplementary Figure 1). Recent studies indicate a prominent role for paracrine EGF production in driving breast cancer metastasis (Wyckoff *et al*., 2004; DeNardo *et al*., 2009). Therefore, we hypothesized that post-EMT, breast cancer cells would be hyperinvasive in response to EGF as compared with pre-EMT cells. Indeed, control 4T07 cells, although highly invasive in response to serum, exhibited little-to-no invasion specifically in response to EGF (Figure 1e). In contrast, post-EMT 4T07 cells readily invaded in response to a solitary EGF stimulus (Figure 1e). Furthermore, and consistent with the establishment of paracrine EGF signaling axes in regulating breast cancer metastasis (Wyckoff *et al*., 2004), we observed significantly elevated quantities of post-EMT 4T07 cells in the lungs of mice at 2 and 4 weeks post-engraftment onto the mammary fat pad (Figure 1f). Although the ultimate fate of disseminated post-EMT cells cannot be ascertained from this experiment, our findings nevertheless show the (1) importance of EMT to enhance metastatic seeding and (2) the inability of EMT to sustain secondary tumor growth (Figure 1f). Consistent with these findings, epithelial-type and nonmetastatic human MCF-7 and MCF10AT1K cells failed to invade in response to EGF, whereas their mesenchymal-type and malignant human

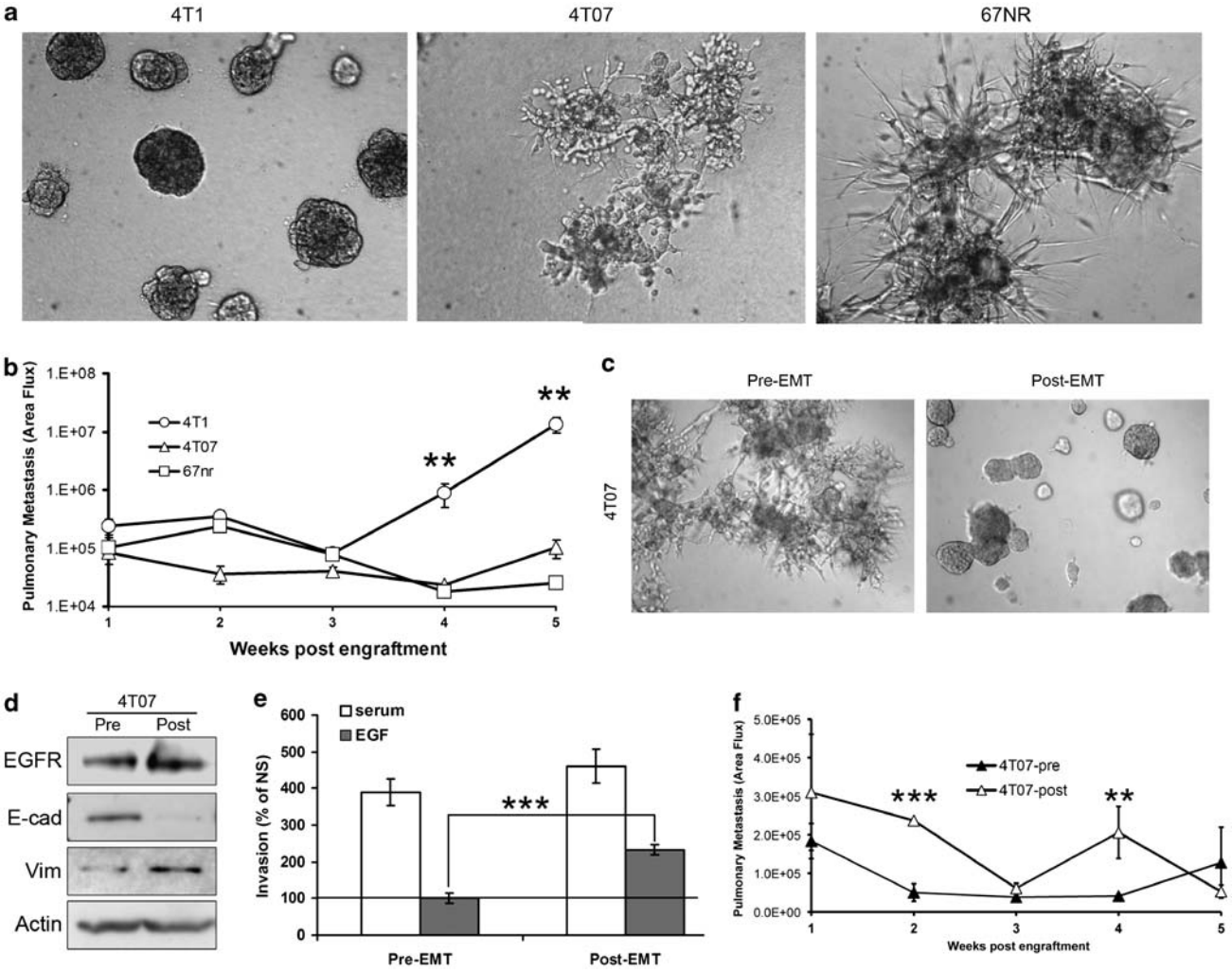

Figure 1 Loss of mammary branching characterizes increasing metastatic potential and is induced by TGF-β1. (**a**) 4T1 (highly metastatic), 4T07 (invasive, but nonmetastatic) and 67NR (noninvasive and nonmetastatic) mammary carcinoma cells were grown in 3D culture for 5 days and representative structures are shown. Data indicate that branched mammary structures are characteristic of nonmetastatic cells, a phenotype that is abandoned by fully metastatic cells. (**b**) The cells described in panel (**a**) expressing firefly luciferase were engrafted onto the mammary fat pad of 4-week-old female Balb/C mice (4T1 = 10 000 cells; 4T07 and 67NR = 100 000 cells). Mean pulmonary luminescence (area flux) is shown at various time points as a measure of metastasis from the primary mammary tumor to the lungs ($n = 5$ mice per group, ± s.e., $^{**}P < 0.01$). (**c**) Pre- and post-EMT 4T07 cells were cultured in 3D-organotypic conditions for 5 days and representative structures are shown. (**d**) The pre- and post-EMT 4T07 cells were analyzed by immunoblot for EGFR, E-cadherin (E-cad), vimentin (vim) and β-actin (actin) as a loading control. Shown are representative immunoblots that were completed three times with similar results. (**e**) Pre- and post-EMT 4t07 cells were allowed to invade through Matrigel-coated synthetic membranes in response to 2% serum or EGF. Data are normalized to a serum-free control (solid line at 100%) and are the mean (± s.e.) of three independent experiments completed in triplicate ($^{***}P < 0.001$). (**f**) The pre- and post-EMT 4T07 cells as shown in panel (c) were engrafted onto the mammary fat pad of Balb/C mice as shown in panel (**b**). Mean pulmonary luminescence (area flux) is shown at various time points following engraftment ($n = 5$ mice per group, ± s.e., $^{***}P < 0.001$, $^{**}P < 0.01$).

MDA-MB-231 and MCF10A-CA1h counterparts readily invaded in response to EGF (Supplementary Figure 2). Collectively, these findings strongly suggest that the process of EMT facilitates the ability of human breast cancer cells to invade aberrantly in response to EGF.

TGF-β stimulation of EMT results in the generation of highly invasive spheroids that possess elevated cell-surface EGFR levels

We next used normal murine mammary gland (NMuMG) cells to more appropriately address the hypothesis that EMT stimulated by TGF-β was sufficient to engender MECs with the ability to invade in response to EGF (Miettinen *et al*., 1994). Figure 2a shows that NMuMG cells endogenously expressed moderate levels of EGFR, which were readily activated along with ERK1/2 in response to EGF (Figure 2a). We demonstrated the specificity of these responses to stimulation by EGF by treating the cells with (1) the Src inhibitor PP2, which uncoupled EGF from phosphorylating EGFR on Y845, but was without effect on EGFR autophosphorylation (Y1116) and ERK1/2 activation; and (2) the EGFR inhibitor AG1478 (AG), which prevented all three EGF-promoted responses (Figure 2a). However, despite their expression of functional EGFR, NMuMG cells did not

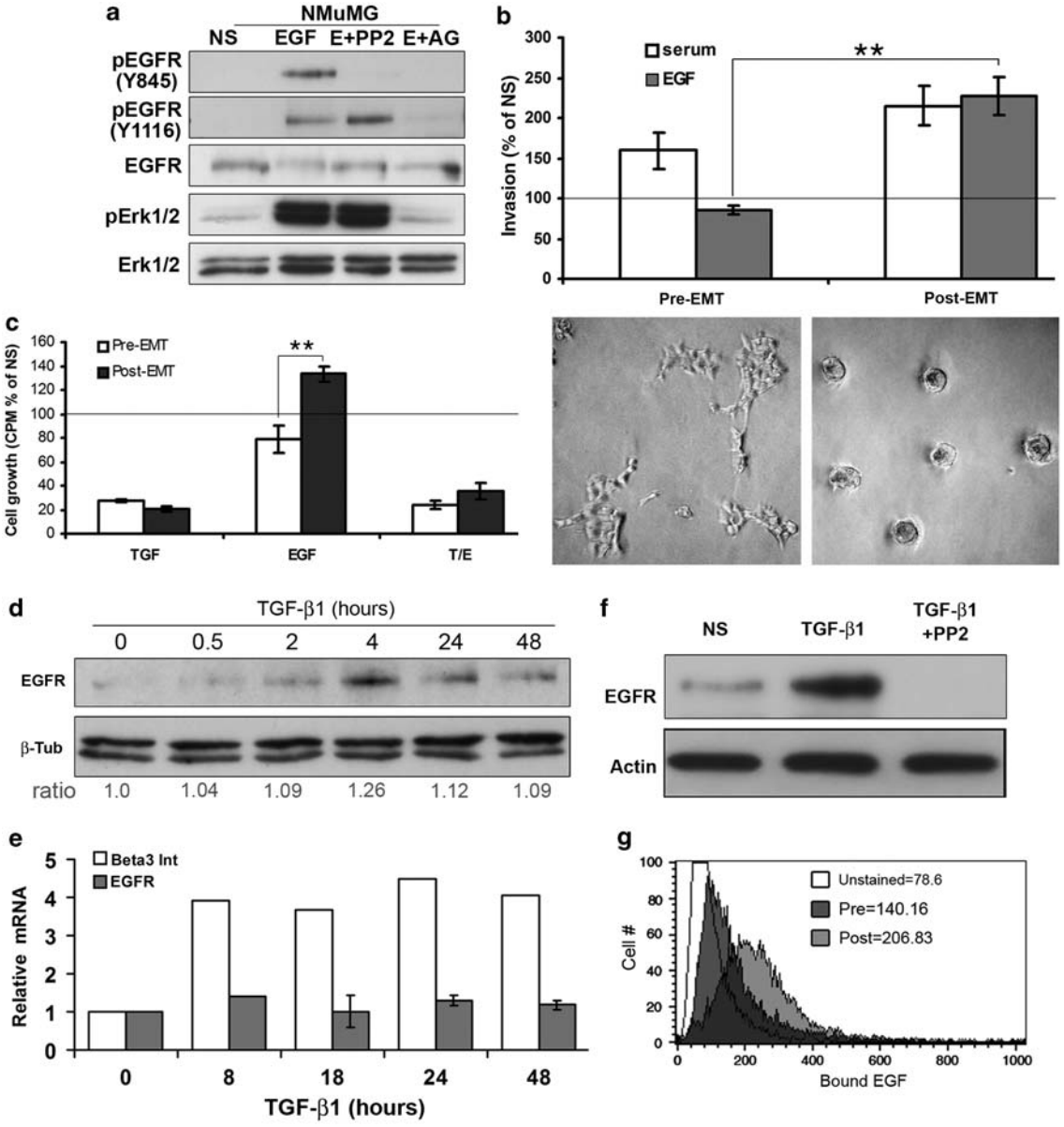

Figure 2 TGF-β stimulation of EMT results in the generation of highly invasive spheroids that possess elevated EGFR cell-surface expression. (**a**) NMuMG cells were serum deprived (0.5% FBS) for 6 h in the absence or presence of the Src inhibitor, PP2 (10 μM) or the EGFR inhibitor, AG1478 (1 μM), at which point they were stimulated for 30 min with EGF and analyzed for the phosphorylation of EGFR (pEGFR(Y845) or pEGFR(Y1116)) or ERK1/2 (pErk1/2). The resulting immunoblots were stripped and reprobed with antibodies against EGFR or ERK1/2, to monitor differences in protein loading. Images are from a representative experiment that was performed at least three times with similar results (NS, no stimulation). (**b**) Pre-EMT and post-EMT NMuMG (48 h pretreatment with TGF-β1 (5 ng/ml)) cells were induced to invade synthetic basement membranes by either serum (2%) or EGF (50 ng/ml) as indicated. Data are the mean (± s.e.) invasion relative to serum-free media for both pre- and post-EMT cells (solid line) observed in three independent experiments completed in triplicate (**$P<0.01$). Accompanying photomicrographs depict the morphology of pre-EMT (lower left) and post-EMT (lower right) NMuMG cells when propagated for 24 h on Matrigel cushions. (**c**) Pre-EMT and post-EMT NMuMG cells were incubated with either TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines for 24 h before labeling cellular DNA by administration of [^{3}H]thymidine. Data are the mean (± s.e.) quantities of incorporated [^{3}H]thymidine normalized to unstimulated controls (solid line) observed in three independent experiments completed in triplicate (**$P<0.01$). (**d**) Quiescent NMuMG cells were stimulated with TGF-β1 (5 ng/ml) for indicated times over a period of 48 h, at which point alterations in EGFR expression were monitored by immunoblotting. Stripped membranes were reprobed with anti-β-tubulin (β-tub) to monitor differences in protein loading. Images are from a representative experiment that was performed at least four times with similar results. (**e**) Quiescent NMuMG cells were stimulated with TGF-β1 (5 ng/ml) as shown in panel (**d**). Afterward, total RNA was isolated and subjected to semiquantitative real-time PCR to monitor the expression of EGFR or β3 integrin, which served as a marker for EMT induced by TGF-β. Data are the mean (± s.d.) fold changes in gene expression relative to untreated control cells observed in at least three independent experiments. (**f**) Quiescent NMuMG cells were stimulated with TGF-β1 (5 ng/ml) for 18 h in the absence or presence of the Src inhibitor, PP2 (10 μM). Alterations in EGFR expression were monitored by immunoblotting as shown in panel (**d**). (**g**) Cell-surface expression of EGFR in pre-EMT and post-EMT NMuMG cells was determined by flow-cytometric analysis of bound Alexa 488-labeled EGF. The presented histogram is representative of three independent experiments.

invade to EGF before their induction of EMT by TGF-β, which conferred robust invasive activities in response to EGF (Figure 2b). Consistent with what we observed in 4T07 cells, post-EMT NMuMG cells showed an 'invasosphere' morphology when cultured on Matrigel (Figure 2b). Moreover, NMuMG cells only showed a proliferative response to EGF subsequent to their induction of EMT (Figure 2c). However, the growth of pre- and post-EMT NMuMG cell populations remained sensitive to the cytostatic activities of TGF-β (Figure 2c).

We next found that TGF-β stimulation of EMT increased the expression of EGFR proteins, a response that was maximal at 4 h and was maintained throughout the 48-h EMT process (Figure 2d). This response was independent of a change in EGFR mRNA (Figure 2e), but was dependent on the activity of Src (Figure 2f). Finally, whole-cell EGF binding assays suggested that TGF-β-induced EMT stabilized EGFR on the cell surface (Figure 2g). Taken together, these findings suggest that the induction of EMT by TGF-β increases stability of EGFR at the cell surface in transitioning cells, which imparts post-EMT MECs with invasive functions in response to EGF.

EMT increases the coupling of EGFR to p38 MAPK activation by FAK

We next sought to determine the function of FAK in regulating EGF signaling in post-EMT MECs. As shown in Figure 3a, EGF-mediated p38 mitogen-activated protein kinase (MAPK) activation and Src-dependent phosphorylation of EGFR at Y845 were greatly augmented in post-EMT NMuMG cells. Importantly, both of these post-EMT EGF signaling events were completely blocked in NMuMG cells depleted in FAK expression (Figure 3a). Recent findings suggest that TGF-β transactivates the EGFR pathway by an extracellular mechanism involving the protease TNF-alpha converting enzyme (TACE)/ADAM17, whose activation by TGF-β mediates the release of EGF ligands (Wang *et al*., 2008, 2009). Supplementary Figure 3 shows that pharmacological antagonism of TACE/ADAM17 or EGFR had no effect on TGF-β-mediated p38 MAPK activation. Furthermore, Figure 3b shows that constitutively elevating EGFR expression in NMuMG cells failed to affect the coupling of TGF-β to p38 MAPK. Interestingly, this same cellular condition specifically enhanced the coupling of EGF to p38 MAPK, but had no affect on the extent of ERK1/2 phosphorylation induced by EGF (Figure 3b). Taken together, these findings suggest that the activation of p38 MAPK by TGF-β takes place by a FAK/Src pathway, whose activation by TGF-β stabilizes EGFR cell-surface expression and enables its coupling to p38 MAPK.

In further addressing the function of FAK in mediating the ability of EGF to induce the invasion of post-EMT MECs, we observed that NMuMGs depleted in FAK expression fail to undergo invasion to EGF in the post-EMT state (Figure 3c). Furthermore, a pharmacological inhibitor of FAK, PF-562271 (PF-271) (Roberts *et al*., 2008) similarly abrogated the invasion of post-EMT control (YFP) NMuMG cells (Figure 3d). Along these lines, inclusion of small molecule inhibitors against TβR-I (SB431542), p38 MAPK (SB203580) or EGFR (AG) also significantly inhibited the invasion of post-EMT control (YFP) NMuMG cells to EGF (Figure 3d). Finally, in light of the elevated expression of EGFR in post-EMT NMuMG cells (Figure 2), we repeated these pharmacological analyses in EGFR-expressing NMuMG cells. Figure 3e shows that constitutive EGFR expression was sufficient to induce invasion to EGF, a cellular reaction that was drastically potentiated in the post-EMT state. As described above, pharmacological inhibition of FAK abrogated the pre- and post-EMT invasion of EGFR-expressing NMuMG cells to EGF (Figure 3e). In stark contrast to their control (YFP) counterparts (Figure 3d), treating post-EMT EGFR-expressing NMuMG cells with inhibitors against either TβR-I or p38 MAPK failed to affect invasion elicited by EGF (Figure 3e). Taken together, these findings suggest that elevated EGFR expression that typically occurs in metastatic breast cancers (Tischkowitz *et al*., 2007) is sufficient in (1) stabilizing the EMT phenotype, (2) allowing persistent invasion to EGF and (3) conferring resistance to small molecules inhibitors against TβR-I and p38 MAPK.

EGFR overexpression transforms NMuMG cells and sensitizes them to EMT by altering EGFR complexes

Given the profound impact constitutive EGFR expression had on EMT-induced invasion to EGF, we next sought to use this NMuMG cell model to further characterize the potential role of EMT in facilitating the ability of EGF to induce breast cancer invasion and metastasis. In doing so, we observed constitutive EGFR expression to be sufficient in transforming NMuMG cells, enabling their production of mammary fat-pad tumors that were comparable with those formed by NMuMG cells engineered to express polyoma middle T (PyMT; Figure 4a). Interestingly, the expression of PyMT in NMuMG cells induced a mesenchymal morphology consistent with increased levels of EGFR and its binding of EGF (Supplementary Figure 4; Salomon *et al*., 1987). Along these lines, constitutive EGFR expression greatly enhanced the mesenchymal character of NMuMG cells stimulated by either TGF-β, EGF or both cytokines together (Figures 4b and c).

Given our findings that suggested that diminished E-cadherin expression determines breast cancer invasion to EGF (Figure 1), we next sought to identify the affects of TGF-β on the physical interaction between EGFR and E-cadherin (Wilding *et al*., 1996; Bremm *et al*., 2008). Figure 4d shows that short-term TGF-β stimulation was unable to affect the expression of E-cadherin or EGFR in confluent cultures (bottom panel). However, we did observe TGF-β to disband the tonic interaction between EGFR and E-cadherin in favor of forming EGFR/TβR-II signaling complexes. Moreover, constitutive EGFR expression alone was sufficient in eliciting a stronger interaction between EGFR and TβR-II that

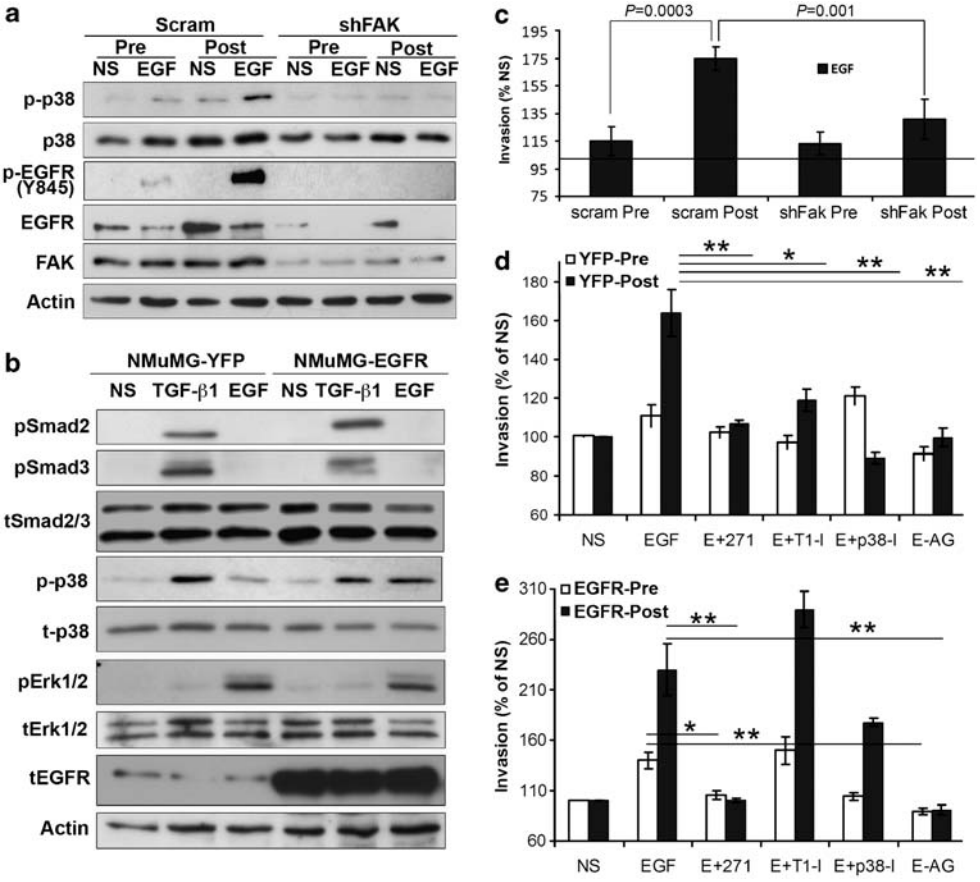

Figure 3 EMT increases the coupling of EGFR to p38 MAPK activation by FAK. (**a**) Control (i.e., scrambled shRNA; scram) and FAK-depleted (shFAK) NMuMG cells were incubated in the absence (pre-EMT) or presence of TGF-β1 (5 ng/ml; post-EMT) for 48 h, at which point they were washed, serum-deprived for 6 h and subsequently stimulated with EGF (50 ng/ml) for 30 min and analyzed for phospho-p38 MAPK (p-p38) or phospho-Y845-EGFR (p-EGFR(Y845)) as indicated. The resulting immunoblots were stripped and reprobed with antibodies against p38 MAPK, EGFR, FAK and β-actin (actin) to monitor differences in protein loading. Images are from a representative experiment that was performed four times with similar results (NS, no stimulation). (**b**) Quiescent control (i.e., YFP) and EGFR-expressing NMuMG cells were stimulated for 30 min with either TGF-β1 (5 ng/ml) or EGF (50 ng/ml) and analyzed to monitor the phosphorylation status of Smad2 (pSmad2), Smad3 (pSmad3), p38 MAPK (p-p38) and ERK1/2 (pErk1/2). The resulting immunoblots were stripped and reprobed for total Smad2/3 (tSmad2/3), p38 MAPK (t-p38), ERK1/2 (tErk1/2), EGFR and β-actin (actin) as loading controls. Images are from a representative experiment that was performed at least four times with similar results. (**c**) Control (i.e., scrambled shRNA; scram) and FAK-depleted pre- and post-EMT NMuMG cells were allowed to invade synthetic basement membranes in response to EGF (50 ng/ml). Data are the mean (± s.e.) invasion relative to unstimulated MECs (i.e., serum-free media placed in the bottom chamber, solid line set to 100%) observed in three independent experiments completed in triplicate. (**d**) Pre- and post-EMT control (i.e., YFP) NMuMG cells were induced to invade EGF (50 ng/ml) in the absence or presence of the following pharmacological inhibitors: FAK inhibitor, PF-562271 (271, 1 μM), TβR-I inhibitor, SB431542 (T1-I, 10 μg/ml), p38 MAPK inhibitor, SB203580 (p38-I, 10 μM), or EGFR inhibitor, AG1478 (AG, 1 μM). (**e**) Data consist of the identical invasion experiments as in panel (**d**) completed with the EGFR-expressing (EGFR) NMuMG cells. Presented data in panels (**d**) and (**e**) are the mean (± s.e.) invasion relative to an EGF-free control (i.e., serum-free media placed in the bottom chamber = NS, set to 100%) observed in three independent experiments completed in triplicate (*$P<0.05$; **$P<0.01$).

mirrored the disassociation of E-cadherin from EGFR, an effect that was further exacerbated by TGF-β treatment (Figure 4d). Importantly, the formation of TβR-II/EGFR complexes correlated with increased Smad2/3 transcriptional activity and maintained cytostatic response to TGF-β (Supplementary Figure 5). To further explore the relationship between EGFR and E-cadherin, we performed immunofluorescent analyses to monitor changes in their expression and localization in NMuMG cells before and after TGF-β-stimulated EMT. E-cadherin expression was indeed decreased and delocalized from cell–cell junctions in EGFR-expressing NMuMG cells as compared with their control counterparts, findings that were exacerbated upon TGF-β stimulation (Figure 5a). Along these lines, Figure 5b clearly shows an emergence of $EGFR^{high}$/$E\text{-cadherin}^{low}$ post-EMT NMuMG cells. Moreover, the most morphologically 'mesenchymal' MECs were completely devoid of both E-cadherin and EGFR expression (Figure 5b; Supplementary Figure 6). Taken together, our findings point to the emergence of a post-EMT breast cancer cell population that is E-cadherin-negative, EGFR-positive and poised to exhibit hyperinvasive responses to EGF.

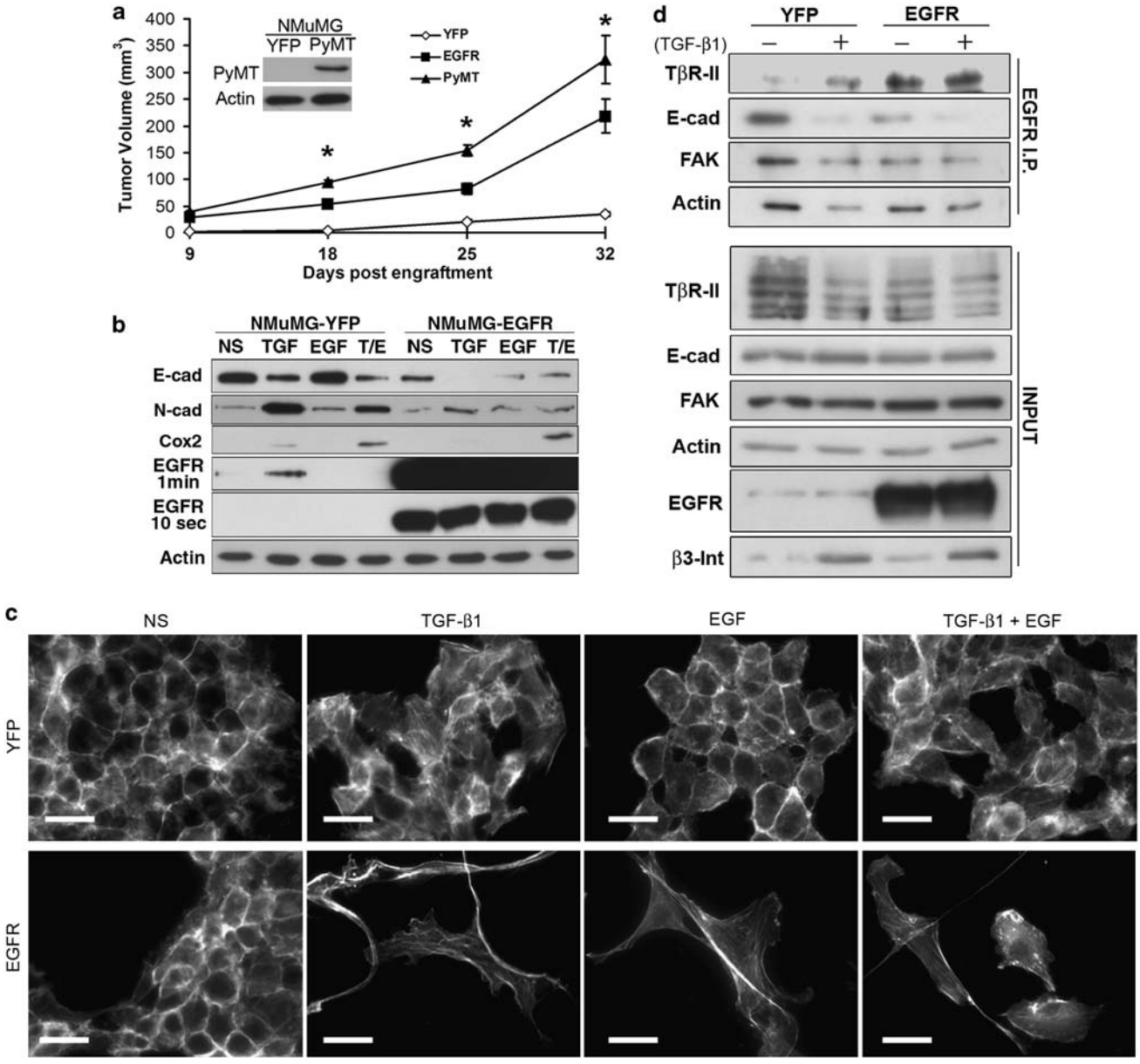

Figure 4 EGFR overexpression transforms NMuMG cells and sensitizes them to EMT by altering EGFR complexes. (**a**) Control (i.e., YFP), PyMT- and EGFR-expressing NMuMG cells were engrafted onto the mammary fat pads of Nu/Nu mice, whose development of mammary tumors was monitored over 32 days. Data are the mean (± s.e.) tumor volumes measured for indicated NMuMG tumor variants. (*$P<0.05$, $n=6$ mice per group). Inset depicts PyMT expression in NMuMG cells, which served as a positive control for tumor formation. (**b**) Control (i.e., YFP) and EGFR-expressing NMuMG cells were stimulated with TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines for 48 h as indicated. Afterward, detergent-solubilized whole-cell extracts were prepared and subjected to immunoblot analyses to monitor changes in the expression of E-cadherin (E-cad), N-cadherin (N-cad), cycloxygenase-2 (Cox2), EGFR and β-actin (actin), which served as a loading control. Images are from a representative experiment that was performed at least three times in its entirety with similar results. (**c**) Control (i.e., YFP) and EGFR-expressing NMuMG cells were stimulated as described in panel (**b**) before visualizing alterations in the actin cytoskeleton by direct phalloidin fluorescence. Images are representative photomicrographs (× 600) from a single experiment that was performed two times with identical results. (**d**) Control (i.e., YFP) and EGFR-expressing NMuMG cells were allowed to reach confluence to normalize E-cad expression levels and incubated in the absence or presence of TGF-β1 (5 ng/ml) for 24 h before isolating EGFR complexes by immunoprecipitation. The resulting EGFR immunocomplexes (EGFR I.P.) were immunoblotted with antibodies against TβR-II, E-cad, FAK and β-actin (actin; top panel). Aliquots of the original whole-cell extract (input) was also immunoblotted with antibodies against TβR-II, E-cad, FAK, β-actin (actin), EGFR and β3 integrin to control for differences in protein loading (bottom panel). Images are from a representative experiment that was performed three times with similar results.

MEC branching induced by EGFR is dependent on TGF-β/FAK signaling

We next sought to evaluate the effects of EMT and constitutive EGFR expression on the growth and morphology of MECs propagated in 3D-organotypic systems. As we noted previously (Wendt *et al*., 2009b), NMuMG cells readily formed organized and hollow acinar structures with a defined actin cytoskeleton when

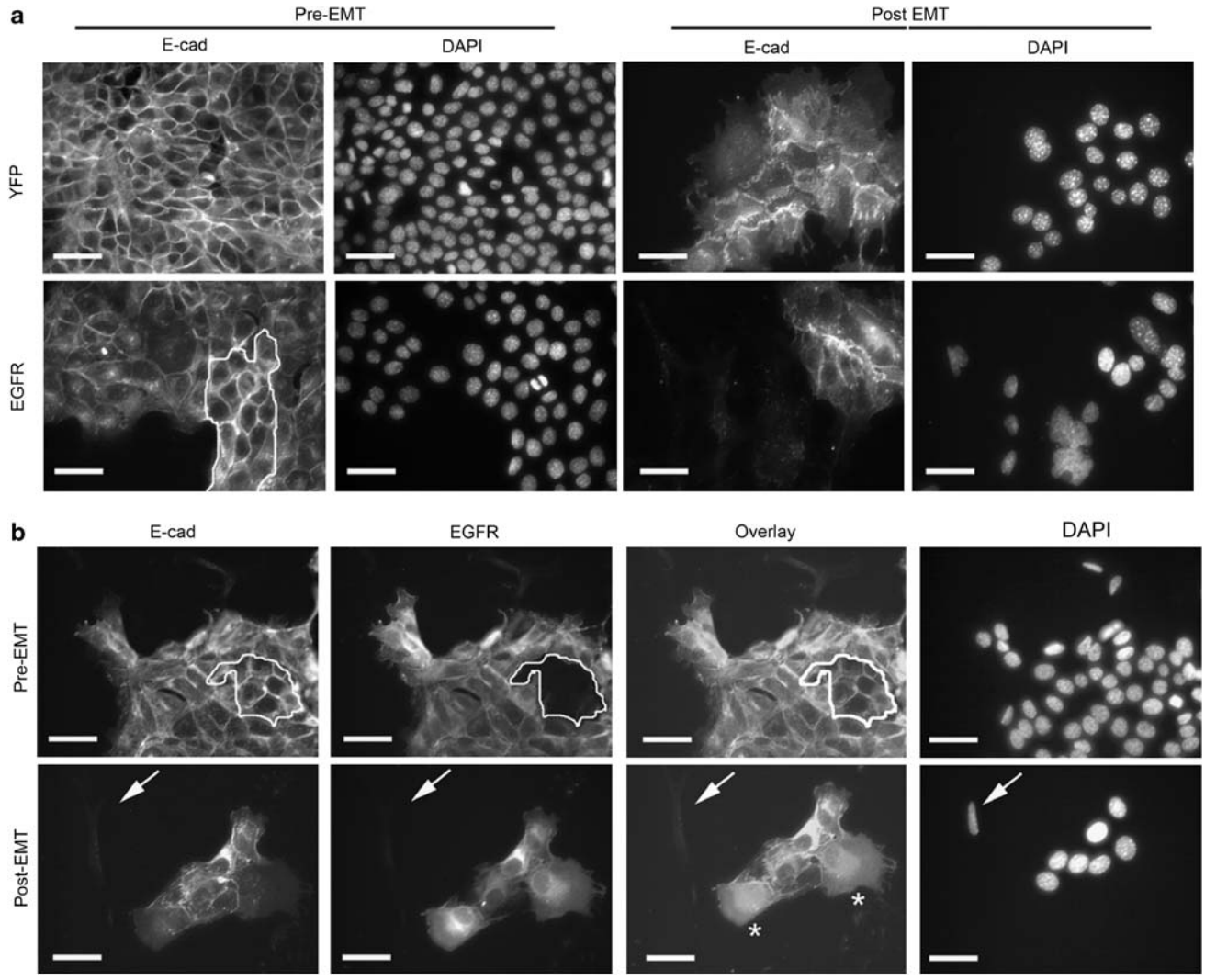

Figure 5 EGFR expression enhanced the delocalization of E-cadherin induced by EMT. (**a**) Control (i.e., YFP) and EGFR-expressing (EGFR) NMuMG cells were incubated in the absence (pre-EMT) or presence (post-EMT) of TGF-β1 (5 ng/ml) 24 h, at which point they were processed for E-cadherin (E-cad) and DAPI immunofluorescence (× 400). Junctional localization of E-cadherin was slightly disrupted in EGFR-expressing NMuMG cells as compared with their control counterparts, except for regional pockets of cells designated by white outline. Delocalized and degradation of E-cadherin in response to TGF-β was enhanced in EGFR-expressing cells. (**b**) EGFR-expressing cells were stimulated to undergo EMT as in panel (**a**), at which point they were subjected to dual immunofluorescent staining to visualize E-cadherin (E-cad, red) and EGFR (green). Regions lacking EGFR expression (white outline) exhibit normal junctional localization of E-cad, whereas EMT induction resulted in the appearance two populations of NMuMG cells: one that was EGFR positive and E-cad negative (*), and a second that lacked expression of both EGFR and E-cad (arrows). Photomicrographs (× 400) presented in panels (**a**) and (**b**) are representative of three independent experiments.

grown in 3D-organotypic conditions (Figure 6a). In stark contrast, and reminiscent of what we observed for nonmetastatic breast cancer cells (Figure 1a), EGFR-expressing NMuMG cells were found to form multicellular and highly branched 3D structures (30.18 ± 6.51% of structures were branched; Figure 6a). These branched organoids may represent an exaggerated activation of normal mammary gland branching activities, particularly since EGF stimulation of parental NMuMG cells also generated branched structures that showed a more differentiated phenotype (48.75 ± 4.71% of structures were branched; Figures 6b and 7b). Pharmacological inactivation of either TGF-β or EGFR signaling completely abrogated mammary branching (0% of structures were branched) and was sufficient in restoring normal, hollow acinar development by EGFR-expressing NMuMG cells (Figure 6b). Chemotherapeutic targeting of FAK (PF-271) prevented mammary branching (0% of structures were branched) and acinar hollowing (0% of structures were hollowed; Figure 6b). Finally, although 3D cultures of post-EMT NMuMG cells failed to elicit any branching structures, this system did produce a significant reduction in acinar hollowing (Figure 6c). Collectively, these findings suggest that the selective appearance of these post-EMT cellular aggregates may similarly represent the hyperinvasive spheroids characteristic of metastatic MECs.

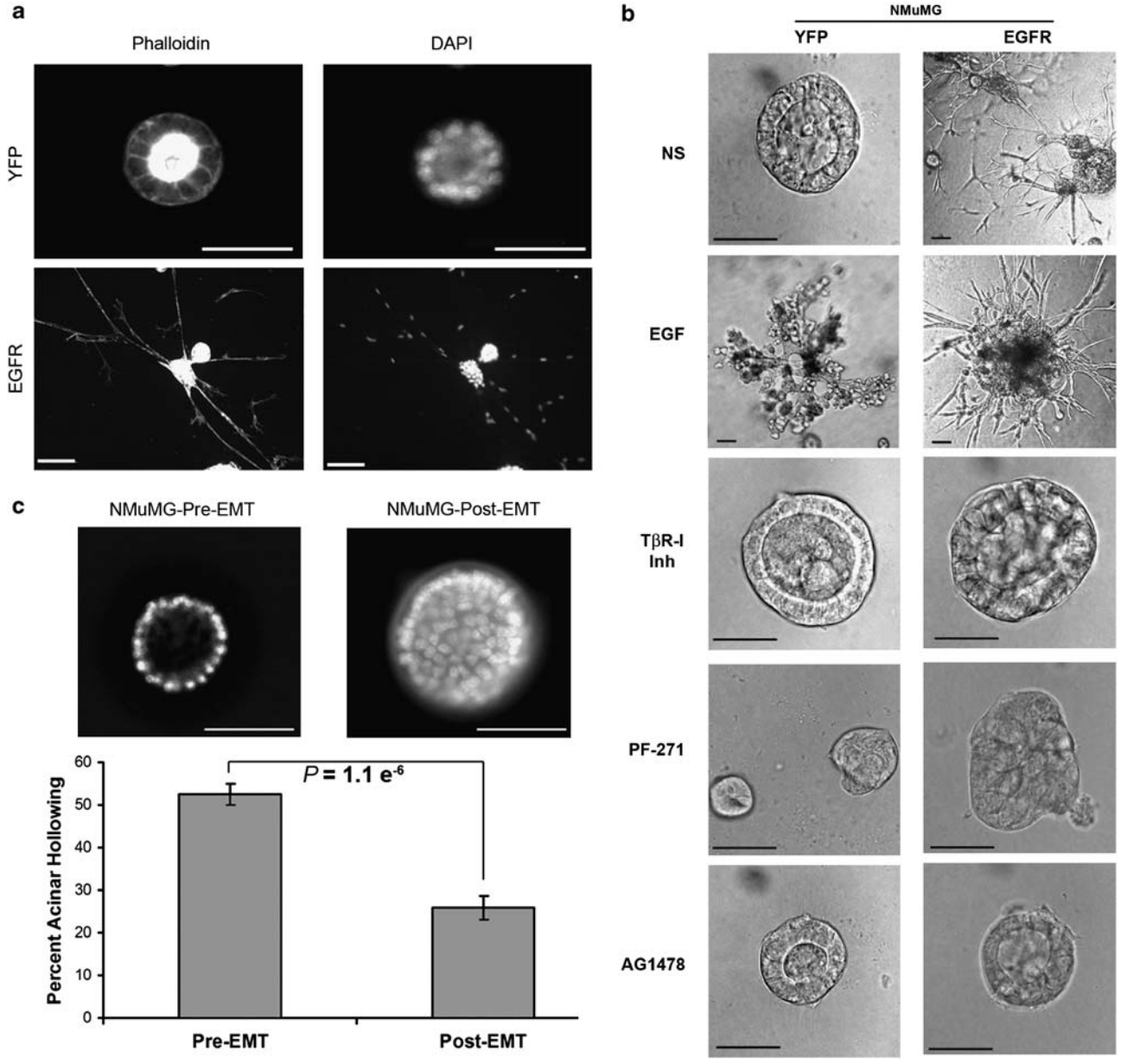

Figure 6 MEC branching induced by EGFR is dependent on TGF-β/FAK signaling. (**a**) Control (i.e., YFP) and EGFR-expressing (EGFR) NMuMG cells were propagated for 10 days in 3D-ogranotypic cultures, and subsequently were processed for direct phalloidin and DAPI fluorescence to visualize the actin cytoskeleton and nuclei, respectively. Shown are representative photomicrographs (YFP = × 400; EGFR = × 100) from a single experiment that was performed more than five times with identical results. (**b**) Control (i.e., YFP) and EGFR-expressing cells were propagated as shown in panel (**a**) in the absence (NS) or presence of either (1) EGF (50 ng/ml); (2) the TβR-I inhibitor, SB431542 (TβR-I inh, 10 μM); (3) the FAK inhibitor, PF-562271 (PF-271, 1 μM); or (4) the EGFR inhibitor, AG1478 (1 μM). Shown are representative photomicrographs (small bar = × 100; large bar = × 400) from a single experiment that was performed atleast three times with similar results. (**c**) Parental NMuMG cells were incubated in the absence (pre-EMT) or presence of TGF-β1 (5 ng/ml; post-EMT) for 48 h before their isolation and propagation for 10 days in 3D-organotypic cultures. Afterward, the resulting organoids were stained with DAPI to visualize the nuclei and the percentage of hollowed acini was quantified. Data are the mean (± s.e.; $n = 3$) percent of hollowed acinar structures. Representative acini are shown.

EMT prevents EGF-induced mammary branching and enhances pulmonary tumor growth

We next aimed to determine which 3D morphology (branching or aggregate spheroid) was dominant under EGF-stimulated conditions. Thus, pre- and post-EMT control (YFP) and EGFR-expressing NMuMG cells were propagated in 3D cultures, supplemented with EGF or the EGFR inhibitor, AG. As observed above (Figure 6b), EGF stimulation of control and EGFR-expressing NMuMG cells readily promoted the formation of normal and dysmorphic branching structures, respectively (Figure 7a). More importantly, Figures 7b and c show that TGF-β stimulation of EMT severely blunted the ability of EGF and EGFR to promote

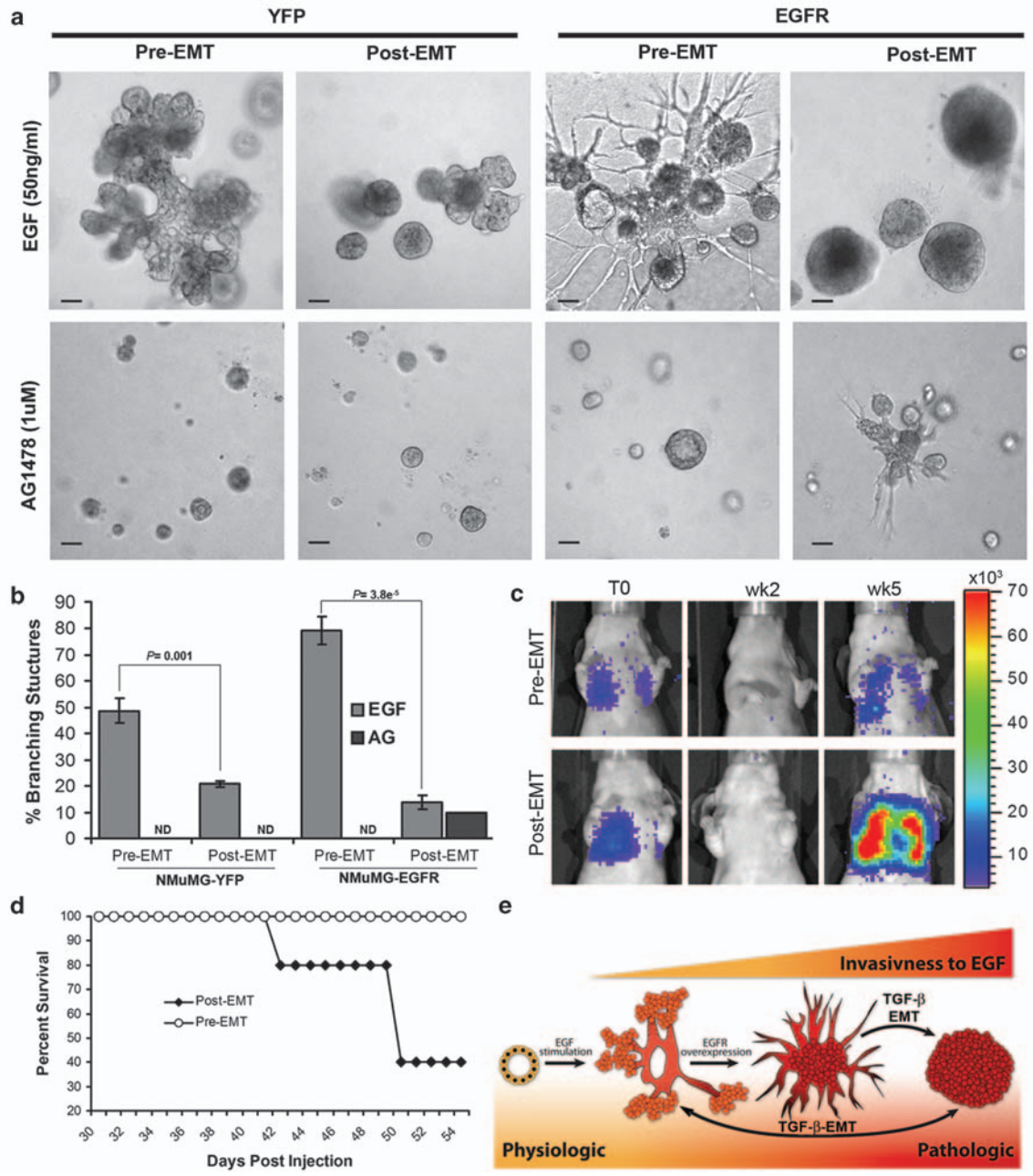

Figure 7 EMT prevents EGF-induced mammary branching and increases pulmonary outgrowth. (**a**) Control (i.e., YFP) and EGFR-expressing (EGFR) NMuMG cells were incubated in the absence (pre-EMT) or presence of TGF-β1 (5 ng/ml; post-EMT) for 48 h before their isolation and propagation for 10 days in 3D-organotypic cultures supplemented with either EGF (50 ng/ml) or the EGFR antagonist, AG1478 (1 μM) as indicated. Differences in organoid morphology were monitored by phase-contrast microscopy (× 100). (**b**) Alterations in organoid branching were quantified and presented as the mean (± s.e.; $n = 3$) percentage of branched structures. (**c**) NMuMG-EGFR cells were transduced with firefly luciferase, treated with TGF-β1 as described in panel (**a**) and injected into the lateral tail vein of 6-week-old, female, Nu/Nu mice. Shown is a longitudinal study of a representative mouse from each group imaged at the indicated time points ($n = 5$ mice per group). (**d**) A survival curve of mice injected with NMuMG-EGFR cells as in panel (**c**), indicating that induction of EMT in NMuMG-EGFR cells decreases the time in which lethal pulmonary tumor burden is reached. (**e**) Schematic depicts the relationship between MEC invasion, their EMT status induced by TGF-β and their 3D culture morphologies. In particular, EMT stimulated by TGF-β suppressed the branching of developing organoids including that induced by EGF/EGFR and instead resulted in the formation of large, dense spheroids that were hyperinvasive to EGF. This unique invasive morphology and phenotype is metastable (double-sided arrow) in normal mammary epithelial cells and required autocrine TGF-β signaling for its manifestation. In stark contrast, post-EMT invasospheres in mammary carcinoma cells are stabilized in their hyperinvasive phenotype (single-sided arrow), and hence rendered independent of the need for continued TGF-β stimulation. Our findings suggest that these novel invasive spheroids likely represent the post-EMT subpopulation of pathologically invasive and metastatic breast cancer cells.

organoid branching, and instead induced the appearance of large, dense cellular aggregates characteristic of metastatic MECs (Figure 1a). Indeed, induction of EMT enhanced pulmonary tumor growth and decreased the survival rate of mice injected with EGFR-transformed NMuMG cells (Figures 7d and e).

Discussion

The increased ability of TGF-β to induce EMT supports the conversion of TGF-β from restraining tumor formation to encouraging their dissemination to distant secondary sites (Tian and Schiemann, 2009; Wendt *et al*., 2009a). Although TGF-β and EGF ligands have a long-standing pathophysiological association with one another, surprisingly little is known about how these signaling systems cross-talk with one another to impact metastasis. Whereas previous reports suggest that TGF-β transactivates EGFR by liberation of EGF ligands (Thomson *et al*., 2008; Wang *et al*., 2008, 2009), we now show for the first time that TGF-β stimulation of EMT elicits a fundamental change in the coupling of EGFR to its downstream effectors. Furthermore, we show that in 3D-organotypic culture post-EMT MECs manifest as dense cellular aggregates that are characteristic of highly metastatic breast cancer cells. Most importantly, we provide evidence that a two-pronged chemotherapeutic approach that targets FAK in conjunction with EGFR specifically inhibited the oncogenic activities of EGF in these aggressive, post-EMT spheroids.

A key regulatory step during EMT is the loss of E-cadherin expression and activity (Yang and Weinberg, 2008; Thiery *et al*., 2009; Wendt *et al*., 2009a). We observed that EMT induced by TGF-β not only downregulated E-cadherin expression but also prevented its interaction with EGFR, allowing for the formation of EGFR/TβR-II complexes that stabilized EGFR at the cell surface of post-EMT MECs. Along these lines, TGF-β has recently been shown to diminish autocrine EGF-ligand production (Thomson *et al*., 2008). Indeed, under these conditions we show that EGFR exhibits heightened availability and responsiveness to paracrine EGF, a signal initiated *in vivo* by reactive tumor stroma (Wyckoff *et al*., 2004). Accordingly, we observed elevated EGFR expression to transform NMuMG cells effectively, as well as to induce their delocalization and downregulation of E-cadherin expression.

Although the overall levels of EGFR were consistently upregulated in parental NMuMG cells undergoing EMT stimulated by TGF-β, we did identify another highly 'mesenchymal-type' population of post-EMT NMuMG cells that lacked EGFR expression. Indeed, a recent study found that prolonged EMT induced by TGF-β (21 days) could elicit cellular switching of receptor tyrosine kinases from a predominantly EGFR-dependent phenotype to one that is dependent on the receptors for fibroblast growth factor or platelet-derived growth factor (Thomson *et al*., 2008). Whether a similar 'switching' mechanism is transpiring in these highly mesenchymal and EGFR-deficient MECs remains to be determined definitively. However, using isogenic cell lines derived from nonmetastatic 67NR cells, we do show for the first time that highly mesenchymal appearing cells cultured on plastic, manifest as dense, cellular spheroids under 3D culture conditions. Indeed, our findings may offer a novel explanation as to why science and medicine routinely fail to identify EMT in human tumors, particularly in metastatic tumor tissue growing in compliant environments like the lungs. Should this prove to be a universal phenomenon, it stands to reason that determining the molecular mechanisms whereby these novel 'invasospheres' undergo invasion seems particularly meritus. In fact, our preliminary analyses suggest that 'invasospheres' can traverse synthetic basement membranes as a single, cooperating unit (data not shown), which contrasts sharply with the initiation of single-cell-based programs of mesenchymal or amoeboid invasion (Friedl and Brocker, 2000).

In addition to our identification of a unique mode of MEC invasion, our *in vitro* protocol of first eliciting EMT in MECs, followed by their subsequent dissociation and subculture in 3D-organotypic systems, in many respects, recapitulates the steps of breast cancer cell metastasis—that is, primary carcinoma cells undergo EMT, exit the primary tumor, survive anoikis in the circulation and finally invade and grow out in a new compliant microenvironment, such as the lung. Indeed, the propagation of breast cancer cells in 3D cultures has been proposed as a model that strongly recapitulates the outgrowth of breast cancer cells in the lung (Shibue and Weinberg, 2009). As depicted in Figure 7e, we propose that breast cancer cells that have undergone EMT abandon their inherent branching program to instead acquire an 'invasosphere' morphology that enables these structures to form large, undifferentiated metastases at distant locales. This model is further supported by our data showing that induction of EMT not only increases primary tumor exit, but also enhances the outgrowth of pulmonary tumors established by tail vein injection.

In attempting to translate our findings to the clinic, it remains to be determined whether the recent inclusion of elevated EGFR expression to the basal-like/triple-negative gene signatures is indicative of the stabilized EGFR phenotype we observed in post-EMT MECs (Tischkowitz *et al*., 2007). However, EMT stimulated by TGF-β does result in the expression of Snail, which subsequently promotes the downregulation of estrogen receptor-α (Dhasarathy *et al*., 2007). Thus, our findings completely support a model in which distinct subpopulations of breast cancer cells undergo EMT, thereby contributing to the development of a gene signature that is indicative of poor clinical outcomes. More importantly, our findings indicate that chemotherapeutic targeting of the TGF-β signaling system that results in its pan-antagonism may offer little-to-no therapeutic benefit in post-EMT MECs that exhibit elevated EGFR expression. Moreover, our analyses also suggest that

simultaneous targeting of FAK in conjunction with EGFR may provide a highly effective means to inhibit these hyperinvasive, post-EMT 'invasospheres.' Experiments designed to test this clinically relevant hypothesis are currently ongoing.

Materials and methods

Cell lines and retroviral reagents

Normal NMuMG cells were obtained from ATCC (Manassas, VA, USA) and cultured as described previously (Galliher and Schiemann, 2007), as was the construction of NMuMG cells that lacked FAK expression (Wendt and Schiemann, 2009). NMuMG cells were engineered to express elevated levels of EGFR by their transduction with VSVG retroviral particles that encoded for either YFP or EGFR (pBabe-YFP or pBabe-EGFR, and provided by Dr Alexander Sorkin (University of Pittsburg, Pittsburg, PA, USA). Afterward, polyclonal populations of transduced NMuMG cells were isolated by puromycin selection (5 μg/ml) for 14 days. In addition, NMuMG cells were also transduced with murine ecotropic viral particles that encoded for either GFP or PyMT (pMSCV-IRES-GFP or pMIG-PyMT-IRES-GFP) and transduced NMuMG cells expressing GFP were isolated by flow cytometry as described previously (Galliher and Schiemann, 2007). The human MCF10A cell derivates T1k and Ca1h were cultured as described previously (Wendt and Schiemann, 2009), as were the conditions necessary to propagate the human MCF-7 (Micalizzi *et al*., 2009), MDA-MB-231 (Wendt *et al*., 2008), 4T1, 4T07 and 67NR (Wendt *et al*., 2009b) cells. Throughout the study, we refer to post-EMT NMuMG cells as those that were stimulated on plastic for 48 h with TGF-β1 (5 ng/ml), whereas post-EMT 4T07 cells were obtained following 3 weeks of continuous TGF-β stimulation through several passages. In all cases, pre-EMT cells represent their unstimulated counterparts.

Cell signaling, immunoblotting and immunoprecipitation assays

To monitor the activation status of TGF-β and EGF effectors, pre-EMT and post-EMT MECs were serum deprived (0.5% fetal bovine serum) for 6 h before their stimulation with TGF-β1 (5 ng/ml) or EGF (50 ng/ml) for varying times as indicated. Afterward, clarified whole-cell extracts were prepared as described previously (Wendt and Schiemann, 2009; Wendt *et al*., 2009b) and subjected to immunoblot analyses using the primary antibodies listed in Supplementary Materials.

In some experiments, confluent cultures of control (YFP) or EGFR-expressing NMuMG cells were treated for 24 h with TGF-β1 (5 ng/ml) before isolating EGFR complexes using previously described immunoprecipitation conditions (Galliher and Schiemann, 2006). The resulting EGFR immunocomplexes (antiEGFR antibodies: 1:100; Cell Signaling, Danvers, MA, USA) were immunoblotted with antibodies against TβR-II, FAK, E-cadherin and β-actin as described above.

Cell biological assays

The inhibition of DNA synthesis by TGF-β was determined using [^{3}H]thymidine incorporation assays as described previously (Wendt *et al*., 2009b). To monitor the ability of EGF to induce DNA synthesis, pre- and post-EMT NMuMG cells were subcultured onto 24-well plates (20 000 cells per well) and allowed to adhere for 4 h, at which point the growth media were removed and replaced with serum-free media supplemented with TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines. After 24 h, the cells were treated with [^{3}H]thymidine, whose incorporation into cellular DNA was quantified as described previously (Wendt *et al*., 2009b).

The ability of EGF (50 ng/ml) or serum (2%) to alter the invasion of pre- or post-EMT cells was analyzed using a modified Boyden Chamber assay (50 000 cells per well) as described previously (Wendt and Schiemann, 2009), in which all values are normalized to a serum-free control set to 100%. In some experiments, the following pharmacological inhibitors were included with EGF throughout the assay: (1) the TβR-I inhibitor, SB431542 (10 μg/ml; EMD Biosciences, San Diego, CA, USA); (2) the FAK inhibitor, PF-562271 (1 μM; Pfizer, Groton, CT, USA); (3) the p38 MAPK, SB230580 (10 μM; EMD Biosciences) or (4) the EGFR inhibitor, AG1478 (1 μM; Cayman Chemical, Ann Arbor, MI, USA).

The ability of TGF-β1 (5 ng/ml), EGF (50 ng/ml) or both cytokines to alter the actin cytoskeleton in NMuMG cells was visualized using TRITC-conjugated phalloidin (0.25 μM; Invitrogen, Carlsbad, CA, USA) as described previously (Galliher and Schiemann, 2006). In some experiments, alterations in the expression and/or localization of E-cadherin and EGFR was monitored by indirect immunofluorescence where the cells were (1) fixed in 4% paraformaldehyde; (2) permeablized in 0.1% Triton X-100 and (3) stained with anti-E-cadherin (1:250) or EGFR (1:100) antibodies. Afterward, E-cadherin immunocomplexes were visualized using biotin-conjugated goat anti-mouse antibodies (Jackson ImmunoResearch, West Grove, PA, USA) in combination with Texas-Red-labeled avidin (Vector Laboratories, Burlingame, CA, USA), whereas EGFR immunocomplexes were visualized simultaneously using FITC-labeled donkey anti-rabbit secondary antibodies (Jackson ImmunoResearch).

EGFR expression analyses

We also monitored alterations in EGFR expression using real-time PCR as described in Supplementary Methods. In addition, pre- and post-EMT NMuMG (1×10^6) cells were resuspended in enzyme-free cell disassociation buffer (Invitrogen), and subsequently incubated in phosphate-buffered saline supplemented with 0.1% bovine serum albumin, to which diluent or Alexa 488-labled EGF (50 ng/ml; Invitrogen) was added. The cells were labeled in the dark and kept on ice for 45 min, then were washed extensively with ice-cold phosphate-buffered saline, fixed in 1% paraformaldehyde and analyzed by flow cytometry.

Tumor growth analyses

Control (YFP), PyMT- and EGFR-expressing NMuMG (2×10^6) cells were resuspended in sterile phosphate-buffered saline (50 μl) that contained 5% Matrigel (BD Biosciences, San Jose, CA, USA), and subsequently injected into the mammary fat pad of 6-week-old female Nu/Nu mice (Charles River, Wilmington, MA, USA). Tumor growth was assessed by calculating primary tumor volumes using digital calipers and the following equation: Tumor Volume $= (x^2)(y)(0.5)$, where x is the tumor width and y the tumor length. Alternatively, pre- and post-EMT NmuMG-EGFR (4×10^5) cells were injected into the lateral tail vein of female Nu/Nu mice and pulmonary tumor growth was monitored by bioluminecent analysis using the Xenogen IVIS200 imager (Xenogen, Alameda, CA, USA) as described previously (Wendt and Schiemann, 2009). Luciferase expressing 4T1 (1×10^4), 4T07 (1×10^5) or 67NR (1×10^5) cells were resuspended in sterile phosphate-buffered saline (50 μl) and engrafted onto the mammary fat pad of 6-week-old female Balb/C mice and pulmonary metastasis was monitored as above. All animal studies were performed

according to animal protocol procedures approved by the Institutional Animal Care and Use Committee of the University of Colorado.

3D-organotypic assays

Pre- and post-EMT NMuMG or 4T07 (1×10^4) cells were resuspended in growth media supplemented with 5% Cultrex (R&D Systems, Minneapolis, MN, USA), and subsequently seeded in 48-well plates onto Cultrex cushions (150 μl per well). In some experiments, acinar development was allowed to transpire in the continued presence of EGF (50 ng/ml), SB431542 (10 μg/ml), PF-562271 (1 μM) or AG (1 μM) as indicated. All NMuMG 3D-organotypic cultures were fed on day 7 and were allowed to develop for an additional 3 days, at which point the extent of their hollowing and branching was assessed by phase-contrast microscopy and quantified by three individuals who were masked to the culture conditions. Where indicated, acinar development also transpired on eight-well chamber slides, which were processed on day 10 for TRITC-conjugated phalloidin (0.25 μM; Invitrogen) and 4′,6-diamidino-2-phenylindole staining as described above. All 4T1, 4T07 and 67NR 3D-organotypic cultures were grown for 5 days and similarly assayed for branching as described above.

Statistical analyses

Statistical values were defined using an unpaired Student's *t*-test, in which *P*-value <0.05 was considered significant. *P*-values for all experiments analyzed are indicated.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

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Transforming Growth Factor-β and the Hallmarks of Cancer

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Abstract

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Tumorigenesis is in many respects a process of dysregulated cellular evolution that drives malignant cells to acquire six phenotypic hallmarks of cancer, including their ability to proliferate and replicate autonomously, to resist cytostatic and apoptotic signals, and to induce tissue invasion, metastasis, and angiogenesis. Transforming growth factor-β (TGF-β) is a potent pleiotropic cytokine that functions as a formidable barrier to the development of cancer hallmarks in normal cells and tissues. Paradoxically, tumorigenesis counteracts the tumor suppressing activities of TGF-β, thus enabling TGF-β to stimulate cancer invasion and metastasis. Fundamental gaps exist in our knowledge of how malignant cells overcome the cytostatic actions of TGF-β, and of how TGF-β stimulates the acquisition of cancer hallmarks by developing and progressing human cancers. Here we review the molecular and cellular mechanisms that underlie the ability of TGF-β to mediate tumor suppression in normal cells, and conversely, to facilitate cancer progression and disease dissemination in malignant cells.

Keywords: Angiogenesis, Apoptosis, Epithelial-mesenchymal Transition, Immunosurveillance, Invasion, Metastasis, Signaling Transduction, Tumor Suppressor

1. Introduction

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Since the inception of the National Cancer Act of 1971, science and medicine have waged an intense battle aimed at conquering cancer. Although considerable progress has been achieved in terms of our understanding of the molecular mechanisms that underlie cancer development and progression, cancer itself remains a significant health concern and burden in the United States. Indeed, 1 in 4 deaths in the United States results from cancer, which also is the leading cause of death in individuals younger than 65 years of age. Despite these grim statistics, overall cancer incidence and mortality rates have begun to decline over the last decade [1]. Continuing along this positive trend will require the development of new diagnostic and chemotherapeutic regimens, as well as the elucidation of new knowledge of how cancer cells acquire the six essential phenotypes, or hallmarks, necessary to become malignant. Included in this phenotypic list is the ability of cancer cells to *(i)* grow autonomously; *(ii)* disregard cytostatic signals; *(iii)* ignore apoptotic signals; *(iv)* stimulate angiogenesis; *(v)* invade and metastasize; and *(vi)* become immortal [2]. Failure by developing neoplasms to acquire each of these phenotypes prevents their conversion to aggressive states, suggesting that these cancer hallmarks

represent various rate-limiting steps during malignant development. As such, pharmacological targeting of cancer hallmarks, both singly and in combination, may offer new inroads to effectively treat the development and dissemination of human malignancies.

Transforming growth factor-β (TGF-β) is a multifunctional cytokine that regulates mammalian development, differentiation, and homeostasis. It is also a potent anticancer agent that prohibits the uncontrolled proliferation of epithelial, endothelial, and hematopoietic cells. Aberrations in the TGF-β signaling pathway bring about resistance to TGF-β-mediated growth arrest, and thus give rise to human malignances [3–5]. Paradoxically, these genetic and epigenetic aberrations conspire to convert TGF-β from a suppressor of tumor formation to a promoter of their growth, survival, and metastasis. Although the molecular details underlying the oncogenic activities of TGF-β remain to be fully elucidated, recent evidence implicates TGF- β as a principle player involved in regulating the acquisition of cancer hallmarks by malignant cells [3–5]. This review focuses on the complex roles played by TGF-β during cancer progression, particularly its ability to regulate the development and progression of malignant cells through each of the individual hallmarks of cancer (Figure 1).

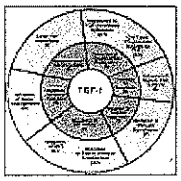

Fig. 1

TGF-β and the hallmarks of cancer. Tumorigenesis converts TGF-β from a powerful tumor suppressor to a lethal tumor promoter that enables evolving cancer cells to acquire the 6 phenotypic traits or hallmarks of cancer. In normal epithelial, ...

2. TGF-β signaling system

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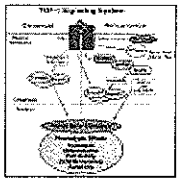
2.1. Canonical TGF-β signaling

The diverse biological activities of TGF-β are mediated through its stimulation of a deceptively simple signaling system that at its core is comprised of three TGF-β receptors, types I (TβR-I), II (TβR-II), and III (TβR-III), and three latent transcription factors, Smads 2, 3, and 4 (Figure 2; [4–8]). The initiation of transmembrane signaling by TGF-β takes place upon its binding to TβR-III, which then presents TGF-β to TβR-II [9]. This ligand presentation mechanism is especially important for TGF-β2, which only interacts with TβR-II when bound to TβR-III [10]. It should be noted that the requirement for TβR-III in propagating messages by TGF-β2 is not absolute, particularly in cells that express a TβR-II variant that binds TGF-β2 independently of TβR-II expression [11]. In contrast, TGF-β1 and TGF-β3 both readily bind to TβR-II and induce intracellular signaling in the absence or presence of TβR-III. The differential requirements of individual TGF-β isoforms for TβR-III, coupled with the spatiotemporal differences observed in their expression and activation patterns [12], likely underlies the more than 30 distinct phenotypes observed in TGF-β1-, TGF-β2-, and TGF-β3-deficient mice [13]. Regardless of its mode of activation, ligand-bound TβR-II subsequently associates with and binds to TβR-I. Both TβR-I and TβR-II house intrinsic Ser/Thr protein kinase activity in their cytoplasmic domains, and the conversion of these ligand:receptor ternary complexes from their inactive to active states requires TβR-II to transphosphorylate TβR-I, thereby stimulating its protein kinase activity [14]. Activated TβR-I in turn stimulates Smads 2 and 3 by phosphorylating these latent transcriptional factors at their C-terminal *SXS* motif. Phosphorylated Smads 2 and 3 undergo a rapid conformational change that facilitates their association with common Smad, Smad4. This conformational change also unveils cryptic nuclear localization sequences in Smads 2, 3, and 4 that promotes heterocomplex accumulation in the nucleus [4–8]. Access to the nucleus enables Smad2/3/4 complexes to interact with an expanding list of transcriptional co-activators and -repressors that collectively alter cell fates through the coordinated induction and repression of TGF-β-responsive genes in a cell- and context-specific manner [4–8].

Fig. 2



Canonical and noncanonical TGF-β signaling. TGF-β stimulates responsive cells by binding and activating two transmembrane Ser/Thr protein kinase receptors termed, TGF-β type I (TβR-I) and type II (TβR-II). Activation ...

Signaling through this canonical TGF-β pathway is regulated and fine-tuned *via* multiple mechanisms that span all cellular compartments. For instance, several adapter/anchoring proteins such as SARA [15], Hgs [16], and Dab2 [17] bind Smad2/3 and facilitate their phosphorylation and activation by TβR-I. This phosphotransferase reaction can be negated by expression of the inhibitory Smad, Smad7, which *(i)* interacts physically with TβR-I and occludes its access to Smad2/3 [18–20], and *(ii)* recruits the E3 ligase Smurf1/2 to facilitate TGF-β receptor ubiquitination and degradation [21, 22]. Moreover, the ability of Smad7 to inhibit TGF-β signaling is augmented by its interaction with STRAP [23], and conversely, is attenuated by its association with either AMSH2 [24] or Arkadia [25–27].

The cellular response to TGF-β also is fine-tuned by the continual nucleocytoplasmic shuttling of Smad2/3, which facilitates their ability to sense and respond rapidly to fluctuations in TGF-β receptor activity [28, 29]. Moreover, regulated phosphorylation of Smad2/3 linker regions is mediated by a variety of protein kinases, including MAP kinases (*i.e.*, ERK1/2 [30], JNK [31], p38 MAPK [32, 33]), Ca^{++}-calmodulin kinase II [34], casein kinase I-ε [35, 36], and CDKs 2 and 4 [37], and is readily reversed by SCP1/2/3-mediated dephosphorylation of Smad2/3 linker regions [38]. At present, the precise role of linker phosphorylation in regulating the biology and pathology of TGF-β action remains to be fully clarified. Similar ambiguity exists concerning the role of sumoylation in regulating the function of Smad4, whose transcriptional activity can be strengthened or weakened by sumoylation in a promoter-dependent manner [39–43]. Finally, termination of Smad2/3/4 signaling takes place primarily through three distinct mechanisms. First, Smad2/3 are rapidly dephosphorylated and inactivated by the nuclear phosphatase PPM1A [44]. Second, Smad2/3/4 undergo polyubiquitination by the E3 ligases Smurf1, Smurf2, and SCF/Roc1 [45–49], leading to their proteosomal degradation. And third, Smad4 activity is suppressed by Ecto/Tifγ, which monoubiquitinates Smad4 on Lys519 and prevents its association with phosphorylated Smad2 [50]. This inhibitory event is readily reversed by FAM/USP9x, which deubiquitinates Smad4 and restores its responsiveness to TGF-β [50].

2.2. Noncanonical TGF-β signaling

Besides its ability to stimulate the canonical Smad2/3 pathway, TGF-β also alters cell behavior through its activation of Smad2/3-independent signaling systems. Included in this growing list of noncanonical effector molecules stimulated by TGF-β are *(i)* the MAP kinases ERK1/ERK2, p38 MAPK, and JNK; *(ii)* the growth and survival kinases PI3K, AKT/PKB, and mTOR; and the small GTP-binding proteins Ras, RhoA, Rac1, and Cdc42 [51–56]. Additionally, activation of the NF-κB signaling system typically is repressed by TGF-β in normal epithelial cells [57]. Recent evidence also implicates TGF-β in mediating the activation of a number of protein tyrosine kinases, including FAK [58, 59], Src [60–63], and Abl [60–62], particularly in mesenchymal or dedifferentiated epithelial cells. Importantly, amplified activation of these noncanonical TGF-β signaling components has been shown to override the normal cellular homeostatic mechanisms governed by Smad2/3 in a manner that figures prominently in the development of human cancers. Precisely how TGF-β couples to the activation of these noncanonical effector systems remains unknown, as does the manner in which their activities becomes dysregulated in response to TGF-β in developing and progressing human cancers. Recent studies linking the inappropriate and/or aberrant activation of alterative TGF-β signaling systems to the acquisition of various cancer hallmarks in developing neoplasms will be presented in the following sections.

2.3. Mutated TGF-β signaling components and cancer

Initial studies aimed at establishing how tumorigenesis negates the cytostatic and tumor suppressing activities of TGF-β were focused primarily on monitoring the expression, mutation, and/or activation status of various

TGF-β signaling components. Indeed, considering the fact that TGF-β is a principle player operant in preventing the uncontrolled growth of epithelial cells, and that ~90% of all human malignancies derive from epithelial cell origins [64], it should come as no surprise to learn that components of the TGF-β signaling system are indeed subjected to frequent mutational inactivation. Moreover, many of these genetic defects are heritable and predispose affected individuals to develop cancer [65, 66]. This scenario is especially evident in colon cancers that exhibit microsatellite instability, which produces frameshift mutations in *TβR-II* and the synthesis of nonfunctional TβR-II proteins that elicit complete cellular insensitivity to TGF-β [67–69]. Clinically, patients with defective TβR-II proteins present with significantly more polyps and preneoplastic lesions than do their TGF-β-responsive counterparts [70]. However, in an interesting twist of fate, patients housing these TβR-II mutants possess a better overall survival rate due to their failure to progress to aggressive, metastatic disease [71]. As will be discussed later, these findings point to an essential role for TGF-β signaling in promoting cancer progression and the acquisition of metastatic phenotypes, particularly in late stage cancers that garner a selective advantage by maintaining their ability to respond to TGF-β. In addition, TβR-II defects also are observed in cancers of the stomach, prostate, breast, lung, liver, and pancreas, and in some hematological malignancies (*see* [3, 4, 72]).

Likewise, diminished expression and/or activity of TβR-I has been correlated with the development of TGF-β resistance in cancers of the colon, pancreas, ovary, breast, cervix, head and neck, and in T and B cell leukemias [3, 72–75]. As a group, TβR-I mutations tend to partition into two general classes, namely those that target its signal sequence [75–77, 78], and those that target its protein kinase domain [79, 80]. Interestingly, an inactivating Ser387Tyr mutation in TβR-I was observed to be more prevalent in metastatic lesions as compared to their corresponding primary tumors, suggesting that this mutation occurs predominantly in late stage cancers where it may play a role in promoting metastasis. Along these lines, cancers of the breast, ovary, prostate, lung, and pancreas frequently lose expression of TβR-III [81–85], an event that enhances the ability of cancer cells to undergo EMT and, consequently, to acquire invasive and metastatic phenotypes. Thus, while TβR-III-deficiency resulting from a loss-of-heterozygousity at or epigenetic silencing of the *TβR-III* locus clearly drives progression of tumors from indolent to aggressive states, the molecular mechanisms underlying the ability of TβR-III to normally suppress these tumorigenic processes remains to be fully elucidated.

Given the importance of Smads 2, 3, and 4 in mediating the tumor suppressing activities of TGF-β, it is not surprisingly to learn that these latent transcription factors also manifest a variety of missense mutations during tumorigenesis. Structurally, Smad2/3/4 are comprised of three distinct domains: *(i)* a globular N-terminal Mad-homology 1 (MH1) domain, which binds TGF-β-regulated promoters at stimulatory **S**mad-**b**inding **e**lements (SBE; GTCTCAGA, CAGA, or AGAC), or at inhibitory **r**epressive SBE (RSBE; GGCGGG) or **T**GF-β **i**nhibitory **e**lements (TIE; GNNTTGGtGa) [8, 86-90]; *(ii)* a central Pro-rich linker region subject to regulated phosphorylation by a variety of protein kinases *(see above)*; and *(iii)* a globular C-terminal MH2 domain, which mediates transactivation through its ability to interact physically with Smad partners, and with various components of the nuclear pore and transcriptional machinery [4, 6, 8]. Most missense mutations identified to date in Smads 2 and 4 localize to the oligomerization sequences within their MH2 domains [91], and to a lesser extent, to the DNA-binding sequences within their MH1 domains [92]. In either scenario, the ability of TGF-β to mediate cytostasis is compromised severely, which enables malignant cells to progress unabated through the cell cycle even in the continued presence of cytokine. This situation occurs frequently in cancers of the gastrointestinal tract, where Smad4 mutations are observed in 50% of pancreatic cancers [66], in 30% of colorectal cancers [3], and in 25% of patients with juvenile polyposis syndrome [3]. Targeted deletion of Smad4 in mouse keratinocytes [93] or T cells [94], or global Smad4 heterozygousity in combination with APC inactivation [95] further confirmed the essential function of Smad4 in suppressing tumor formation, particularly that in the gastrointestinal track. Although the prevalence of Smad2 mutations is considerably less than that observed for Smad4, mutations in Smad2 are detected in 11% of colorectal cancers, and in 7% of lung cancers [3]. Lastly, despite the fact that Smad3 mutations have thus far remained undetected in human cancers, its expression is lost in 37% of human gastric cancers [96], and in essentially all childhood cases of

acute T cell ALL [97]. Furthermore, unlike mice deficient in either Smad2 or Smad4, mice lacking Smad3 are viable and develop colorectal cancers only in response to *Helicobacter* infection and its accompanying inflammatory reaction [98]. More importantly, Smad3-deficiency protects mice from developing chemically-induced skin carcinomas [99]. Collectively, these findings indicate Smad2 and Smad3 mediate distinct aspects of the biology and pathology of TGF-β; they also suggest that retaining selective features of the TGF-β signaling system functions in promoting oncogenic signaling by TGF-β. The molecular mechanisms that underlie oncogenic signaling by TGF-β, as well as their role in achieving the hallmarks of cancers are discussed below.

3. TGF-β and dysregulated cell proliferation

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3.1. Resistance to TGF-β-mediated cytostasis

TGF-β is a principle player involved in suppressing autonomous growth by epithelial, endothelial, and hematopoietic cell lineages, doing so primarily through its ability to induce cell cycle arrest, but also through its stimulation of cell differentiation or apoptosis in a cell- and context-specific manner. Importantly, the ability to preneoplastic cells to acquire resistance to growth arrest governed by TGF-β represents a major hallmark in the development of numerous human cancers. The ability of TGF-β to mediate cytostasis takes place late in the G1 phase of the cell cycle and occurs primarily through the initiation of two synchronized events. First, TGF-β downregulates the expression of the growth-promoting transcription factors, c-Myc and Ids 1–3 [4, 6, 100, 101]. In doing so, TGF-β inhibits Myc transcription by inducing the binding of Smad3 to E2F4/5 and p107 at RSBE sites housed in the Myc promoter [87, 89]. Smad3 also mediates Id1 repression by TGF-β through a "self-enabled" signaling system that first requires Smad3 to induce the expression of ATF3, which subsequently interacts with Smad3 to inhibit Id1 promoter activation [102]. Loss of Id2 expression requires TGF-β to induce the expression of the Myc antagonists, Mad2 and Mad4, which form heterodimers with Max and prevent Id2 transcription [101].

The second major pathway whereby TGF-β mediates cytostasis occurs through its production of the cyclin-dependent protein kinase (CDK) inhibitors, $p15^{INK4b}$ and $p21^{CIP}$ [4, 6, 100]. The ability of p15 to prevent cell cycle progression takes place by its binding to CDK4/6, which occludes their access to and activation by cyclin D. TGF-β induces p15 expression *via* a bimodal mechanism involving *(i)* Smad3-mediated repression of Myc, which together with Miz-1 binds and inactivates p15 transcription; and *(ii)* the formation of Sp1:Smad3:Miz-1 complexes that stimulate p15 transcription [103, 104]. Whereas p15 preferentially inactivates CDK4/6, p21 preferentially targets and antagonizes cyclin E:CDK2 complexes [100]. In doing so, TGF-β stimulates the formation of Sp1:Smad2/3:FoxO complexes that transactivate the p21 promoter [105, 106].

Developing and progressing neoplasms have evolved several mechanisms to override the cytostatic activities of TGF-β. For example, dysregulated Myc expression negates cell cycle arrest induced by TGF-β by *(i)* promoting the formation of Myc:Miz-1 complexes, which inhibit p15 and p21 transcription induced by Smad3:Miz-1 complexes [103, 104]; and *(ii)* recruiting the DNA methyltransferase, Dnmt3a, to Myc:Miz-1 complexes, which methylates and inactivates p21 transcription [107]. In addition, human cancers frequently exhibit hyperactivation of the PI3K/AKT pathway [108], which inactivates the cytostatic activities of TGF-β by *(i)* phosphorylating FoxO, which subsequently is exported from the nucleus and sequestered in the cytoplasm by 14-3-3, and as such, is unavailable to induce p21 expression with Smad3 [105, 109]; and *(ii)* binding and sequestering inactive Smad3 to prevent its stimulation by active TGF-β receptors [110, 111]. Moreover, in conjunction with activated AKT, FoxG1 interacts physically with Smad3:FoxO complexes to inhibit their induction of p21 in glioblastomas [105]. Whether FoxG1 or other Forkhead family members function similarly to inactive p15 and p21 expression in other human cancers remains to be determined definitively.

Cancer cells also evade the cytostatic activities of TGF-β *via* cancer cell-specific alternative splicing of C/EBPβ into transcriptionally active LAP or inactive LIP variants [112]. Under normal circumstances, LAP C/EBPβ variants participate in TGF-β-mediated cytostasis by cooperating with Smad3:FoxO complexes to induce p15 expression, and with Smad3:E2F4/5 complexes to repress Myc expression [112]. Cancer cells, particularly

those of the breast, elevate their expression of inactive LIP C/EBPβ variants, which uncouples TGF-β from regulation Myc and p15 expression and correlates with metastatic disease development [112]. Along these lines, ELAC2 recently was identified as a transcriptional co-factor that mediates p21 expression in conjunction with Smad2:FAST-1 complexes in prostate epithelial cells [113]. Importantly, loss of ELAC2 in developing and progressing prostate cancers inactivates their ability to undergo growth arrest in response to TGF-β [114]. Finally, overexpression or oncogenic activation of the Ski and SnoN induces cellular transformation in part *via* their ability to bind Smad2/3/4 complexes and recruit transcriptional inactivation machinery, including N-Cor, mSin3A, and HDAC, which collectively repress gene induction stimulated by TGF-β [115, 116].

3.2. Cell cycle progression induced by TGF-β

In addition to developing resistance to TGF-β-mediated growth arrest, cancer cells routinely acquire the ability to undergo enhanced proliferation when stimulated by TGF-β. The precise mechanisms underlying this phenomenon has yet to be established, but likely reflects a combination of the inactivation of cytostasis mediated by TGF-β coupled to its ability to induce the expression of cytokines and growth factors or their cognate receptors. For instance, TGF-β stimulates the synthesis of IL-1 [117], CTGF [118], bFGF [119], PDGF [120], and TGF-α [121], and of the receptors for PDGF [122] and EGF [123]. Moreover, a common feature of mitogenic signaling systems is their coupling to activation of the Ras/MAP kinase pathways, which results in ERK1/2-mediated phosphorylation of the linker region of Smad2/3 linker and their exclusion from the nucleus, perhaps *via* Smurf-mediated sequestration of Smad2/3 from the nuclear pore machinery [124]. Hyperactivation of the Ras/MAP kinase pathway also promotes ERK1/2-mediated phosphorylation of TGIF, leading to its stabilization and nuclear localization where it functions as a transcriptional co-repressor by recruiting Smad2:HDAC complexes to the p15 promoter [125]. More recently, activation of RTK and Ras/MAP kinase signaling was shown to stimulate CK1ε/δ phosphorylation of p53, which interacted physically with Smad2/3 to promote the initiation of the cytostatic program by TGF-β. Importantly, restoring p53 function to cancer cells devoid of p53 expression or activity reinstated the ability of TGF-β to induce p21 expression and, consequently, cell cycle arrest [36]. Finally, besides their ability to activate the Ras/MAP kinase pathway, mitogens also activate CDKs when promoting cell cycle progression. In doing so, the cytostatic function of TGF-β can be subverted by CDK2- and CDK4-mediated phosphorylation of the linker region of Smad2, which inactivates its ability to induce the expression of p15 and p21, and to repress the expression of Myc [37].

4. TGF-β and cancer cell motility

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4.1. TGF-β and epithelial-mesenchymal transition

The process whereby immotile, polarized epithelial cells to transdifferentiate into highly motile, apolar mesenchymal cells is known as epithelial-mesenchymal transition (EMT), was described initially as a phenomenon that took place during chick embryonic development nearly 100 years ago by Frank Lillie [126], and which was first documented as a discrete physiological event in 1982 by Greenburg and Hay [127]. Today, EMT is recognized as a normal and essential physiological process operant in mediating embryonic development, wound healing, and tissue morphogenesis, remodeling, and repair. EMT itself compromises several distinct features, including *(i)* the loss of cell polarity due to downregulated expression of epithelial cell markers (*e.g.*, E-cadherin, zona occluden-1, and β4 integrin); *(ii)* cytoskeletal architecture reorganization and intracellular organelle redistribution; *(iii)* upregulated expression of fibroblast markers (*e.g.*, vimentin, N-cadherin, α-smooth muscle actin); and *(iv)* elevated cell invasion and migration [126, 128–131]. Besides its role in mediating normal tissue morphogenesis and repair, inappropriate initiation of EMT also underlies the development of several human pathologies, such as chronic inflammation, rheumatoid arthritis, and chronic fibrotic degenerative disorders of the lung, liver, and kidney [126, 128–132]. In addition, the acquisition of metastatic phenotypes by dedifferentiated tumors is critically dependent on EMT and its ability to actively remodel tumor microenvironments in a manner that promotes the evolution and selection of metastatic cells [126, 128–131].

The essential role of TGF-β in mediating EMT was first described by Miettinen *et al* [133]who showed that TGF -β stimulation of normal mammary epithelial cells readily induced their transdifferentiation into fibroblastoid-like cells. Since that time, TGF-β has been identified as a master regulator both of physiological and pathophysiological EMT in a variety of epithelial cell types and tissues. For instance, TGF-β3-deficiency in mice elicits defective palatogenesis and cleft palate formation due to faulty initiation of EMT [134]. Along these lines, neutralization of TGF-β2 function impairs chick heart endocardial cushion development by inhibiting Slug expression and, consequently, its induction of EMT [135]. Finally, targeted-deletion of Smad3 in mice prevents their development of EMT-driven retinal [136, 137] and renal [138] fibrosis. Aberrant EMT also is an essential facet of the oncogenic activities of TGF-β, particularly its ability to stimulate the progression and metastasis of late stage cancers. The molecular mechanisms underlying the ability of TGF-β to induce cancer cell EMT and metastasis are described below.

4.2. TGF-β, invasion, and metastasis

The activities of fibroblasts and their associated stromal components play important roles in determining whether TGF-β either suppresses or promotes tumor formation [139, 140]. Indeed, the ability of TGF-β to inhibit tumor formation occurs not only through its actions in epithelial cells, but also through its stimulation of adjacent fibroblasts, which synthesize and secrete a variety of cytokines, growth factors, and extracellular matrix (ECM) proteins that mediate tissue homeostasis and suppress cancer development. Importantly, genetic or epigenetic events that inactivate paracrine TGF-β signaling between adjacent epithelial and stromal compartments can lead to the formation of neoplastic cells, as well as to their selection and expansion by promoting their growth, survival, and motility [141, 142]. For instance, conditional inactivation of TβR-II in mouse fibroblasts elicits the formation of prostate intraepithelial neoplasias and invasive carcinoma of the forestomach [143]. Similar targeted inactivation of TβR-II in mouse mammary fibroblasts inhibits mammary gland ductal morphogenesis, as well as the formation of terminal end buds [144]. Importantly, grafting mammary carcinoma cells with TβR-II-deficient fibroblasts, not their normal counterparts, significantly enhanced the growth and invasion of breast cancer cells in subrenal capsules [144]. Mechanistically, inactivation of TGF-β signaling in fibroblasts promotes tumorigenesis by upregulating the expression and signaling of TGF-α, MSP, and HGF within cell microenvironments [139, 140, 143, 144]. Furthermore, inactivating TGF-β signaling in fibroblasts also promotes breast cancer metastasis by activating two chemokine receptor axes, namely SDF-1:CXCR4 and CXCL5:CXCR2, which recruit immature GR1+CD11b+ myeloid cells that *(i)* suppress host tumor immunosurveillance, and *(ii)* induce MMP expression within tumor microenvironments that promotes the dissemination of breast cancer cells [145].

In addition to its metastatic-promoting activities initiated in fibroblasts and tumor microenvironments, TGF-β also induces EMT and metastasis by directly effecting the activities and behaviors of developing and progressing malignant cells. For instance, TGF-β stimulates human MDA-MB-231 breast cancer cells to metastasize specifically to bone by inducing their expression of PTHrP, IL-11, and CTGF [146–148]. Interestingly, although TGF-β1 had no effect on the growth of mammary tumors induced by transgenic polyomavirus middle T antigen expression, conditional TGF-β1 expression significantly enhanced the ability of these same mammary tumors to metastasize to the lungs [149]. In addition, TGF-β stimulation of EMT in breast cancer cells has been linked to the selection and expansion of cancer initiating/stem cells [150–153], which may underlie breast cancer resistance to neoadjuvant chemotherapies, as well as disease progression and recurrence. Thus, following inactivation of its cytostatic function, TGF-β preferentially promotes the acquisition of metastatic phenotypes in previously nonmetastatic malignant cells.

Accumulating evidence indicates that TGF-β stimulates cancer cell EMT and metastasis through a combination of Smad2/3-dependent and -independent signaling systems. Indeed, engineering metastatic human MCF10ACA1a breast cancer cells to express a dominant-negative Smad3 [154] or a TβR-I mutant incapable of activating Smad2/3 (*i.e.*, L45 mutant) [154] both significantly reduced the ability of MCF10ACA1a cells to colonize the lung. Along these lines, Smad4-deficiency elicited by RNA interference inhibited the ability of

MDA-MB-231 xenografts to metastasize to bone in part *via* diminished expression of IL-11 and CTGF in response to TGF-β [146, 155]. As above, the extent of tumor formation and their rate of growth was unaffected by altering the response of these breast cancer cells to TGF-β [146, 155], which again points to the importance of maintaining the fidelity of the TGF-β signaling system during metastasis development. Accordingly, whereas Smad4-deficiency conspires with oncogenic K-Ras to promote pancreatic cancer initiation and development, maintaining intact Smad4 signaling is necessary to induce EMT and TGF-β-dependent growth of advanced pancreatic ductal adenocarcinomas [156]. In addition, overexpression of Smad7, which inhibits TGF-β stimulation of Smad2/3 [18–20], prevents the invasion of breast [157] and head and neck cancers [158, 159], as well as impairs the ability of melanoma cells to metastasize to bone [160]. Recently, TGF-β was shown to cooperate with Ras to promote the formation of mutant p53/p63 complexes that are bridged by Smad2/3, and that result the inactivation of p63 and its ability to downregulate the expression of metastasis suppressor genes, Sharp-1 and cyclin G2 [161]. Along these lines, Smad2 has been observed to promote EMT in mammary epithelial cells by stimulating the DNA binding activity of the DNA methyltransferase, DNMT1, leading to the chronic epigenetic silencing of epithelial-associated genes, including *CDH1*, *CGN*, *CLDN4*, and *KLK10* [162]. Collectively, these findings demonstrate the importance of Smad2/3/4 signaling in mediating metastasis stimulated by TGF-β; they also suggest that the development and implementation of novel Smad2/3 antagonists may improve the clinical course of metastatic cancer patients whose tumors house functional Smad2/3 signaling systems.

Equally important to the ability of TGF-β to stimulate cancer cell EMT and metastasis is its coupling to activation of noncanonical signaling systems (Fig. 2; [7]). Indeed, activation of Ras/MAP kinase [51, 163–167], PI3K/AKT [52], Rho/ROCK [53]; NF-κB [168], Jagged/Notch [169], Wnt/β-catenin [170], and MDM2/p53 [171] pathways by TGF-β all play essential roles in mediating its induction of cancer cell EMT, invasion, and metastasis. In particular, Ras/MAP kinase signaling cooperates synergistically with TGF-β to elicit EMT and metastasis during the progression of skin cancers from squamous to spindle cell phenotypes [172–174]. In addition, TGF-β stimulation of NF-κB promotes EMT in and lung colonization by breast cancer cells that house oncogenic Ras [168]. Furthermore, activation of the Ras effector, c-Raf-1, induces autocrine TGF-β expression and activation that ultimately results in its stimulation of EMT and invasion [175]. Finally, TGF-β-induces elevated MDM2 expression that leads to the destabilization of p53, a key component of EMT [171].

Integrins also have been implicated in regulating oncogenic signaling by TGF-β, particularly its ability to induce EMT, invasion, and metastasis. For instance, administering neutralizing antibodies against β1 integrin prevented TGF-β stimulation of p38 MAPK and EMT in mammary epithelial cells [163]. In addition, TGF-β stimulates the binding of the adapter protein Dab2 [17] to β1 integrin, leading to FAK activation and the induction of EMT in mammary epithelial cells [176]. Similarly, work in our laboratory led to the discovery of a novel αvβ3 integrin:Src:phospho-Y284-TβR-II:Grb2:p38 MAPK signaling axis whose activation mediates oncogenic signaling – *i.e.*, EMT, invasion, and metastasis – by TGF-β in normal and malignant mammary epithelial cells [60–62]. Importantly, the ability of TGF-β to stimulate the growth and pulmonary metastasis of breast cancers in mice absolutely requires activation of this oncogenic signaling complex [62]. In addition to its role in mediating pulmonary metastasis of breast cancers, αvβ3 integrin also mediates breast cancer metastasis to bone [177, 178] in a TGF-β-dependent manner. Lastly, new insights into the role of TGF-β in regulating tight junction dissolution during EMT recently was elucidated by Wrana and colleagues [179] who observed that the tight-junction assembly protein, PAR-6, interacts physically with TβR-I. Activation of TGF-β receptors by ligand results in TβR-II-mediated phosphorylation of PAR-6, which binds Smurf1 and coordinates its ubiquitination and degradation of RhoA. The net effect of these TGF- β-dependent events results in the dissolution of epithelial cell tight junctions and the disassembly of their actin cytoskeleton, leading to the induction of EMT [179]. Future studies need to more thoroughly examine the role of additional phosphorylation events in potentially regulating PAR-6 function, as well as the role of integrins and other adhesion-regulated signaling systems to collaborate with TGF-β in mediating EMT and tight junction

dissolution. Similar analyses aimed at determining the importance of these events in regulating metastasis development stimulated by TGF-β also is warranted.

5. TGF-β and tumor angiogenesis

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Angiogenesis is a normal physiological process whereby new blood vessels develop from preexisting vessels, and which is an essential component of embryonic development, wound healing, and the female reproductive cycle [180, 181]. Pathological activation of angiogenesis also figures prominently in mediating the development of a number of human diseases, including rheumatoid arthritis, diabetic retinopathy, and age-related macular degeneration [181, 182]. Indeed, inappropriate initiation of angiogenesis perhaps is best known and characterized for its role in mediating the development and progression of tumors, whose growth is limited by the extent of nutrient diffusion into tumor microenvironments. Tumor angiogenesis circumvents this limitation by providing developing neoplasias with an efficient supply of nutrients, and ultimately, with a route for their metastatic spread. Angiogenesis is comprised of two distinct and sequential phases referred to as angiogenesis activation and angiogenesis resolution [180–182]. Angiogenesis activation encompasses the initiation and development of new blood vessels, and is characterized by *(i)* increased endothelial cell (EC) permeability, proliferation, migration, and invasion, and *(ii)* reduced EC adhesion and basement membrane integrity. In contrast, angiogenesis resolution encompasses the maturation of newly formed vessels and entails *(i)* increased EC adhesion and basement membrane deposition, *(ii)* decreased EC proliferation and motility, and *(iii)* the recruitment of perivascular cells, namely pericytes, vascular smooth muscle cells, or mural cells, that function in regulating vessel stability and hemodynamics. Importantly, normal endothelium in adult tissues exists in a quiescent, inactive state, a fact that underlies the continued enthusiasm to selectively target tumor vascular when treating human malignancies.

Consistent with its multifunctional nature, TGF-β has been reported to regulate the activation and resolution phases of angiogenesis [183–186]. In addition, TGF-β also affects ECM production and remodeling in EC microenvironments, and as such, impacts the interactions and communications between ECs and their supporting mesenchymal cells [187]. The ability of TGF- β to regulate angiogenesis was discovered by analyzing the vasculature of TGF-β1-deficient mice, which exhibit severe defects in hematopoiesis and EC differentiation that results in the production of weak, aberrantly formed capillaries [188]. Additional studies demonstrating a crucial role of TGF-β signaling in regulating angiogenesis were gleaned from analyses of mice lacking TβR-I [189], TβRII [190, 191], TβR-III [192, 193], ALK1 [194, 195], endoglin [196], Smad1 [197], or Smad5 [198], all of which exhibited vascular and endothelial defects. Clinically, the development of hereditary hemorrhagic telangiectasia type 1 (HTT1) results from the loss or inactivation of the gene for endoglin [199, 200], while the loss or inactivation of the gene for ALK1 elicits HHT2 [201, 202]. Moreover, HHT1 and HHT2 both are phenocopied in knockout mice lacking either endoglin [196, 203] or ALK1 [204], respectively. Thus, aberrant TGF-β signaling clearly underlies the development of multiple vascular disorders [205].

Genetic inactivation of ALK1 [195] and ALK5 [189] elicits embryonic lethality at E11.5 and E10.5, respectively, demonstrating the indispensable function of these genes in mediating normal embryonic angiogenesis and vasculogenesis. Quite surprisingly, recent studies have established that TGF-β differentially activates TβR-I/ALK5 *versus* ALK1, which elicits dramatically different angiogenic responses by ECs. For instance, TGF-β stimulation of TβR-I/ALK5 activates Smad2/3 and their transcription of the ECM proteins, plasminogen activator inhibitor type 1 (PAI-1) and fibronectin, which mediate angiostasis and vessel maturation [194, 206, 207]. In addition, Miyazono and colleagues [208] showed that TβR-I/ALK5 mediates TGF-β stimulation of EC gene expression profiles characteristic of angiostasis and vessel maturation, and of periendothelial cell differentiation. Thus, activation of TβR-I/ALK5 by TGF-β functions preferentially in regulating angiogenesis resolution [185, 208]. In contrast, activation of ALK1 by TGF-β stimulates Smad1/5/8 transcriptional activity and the expression of angiogenic genes, such as Id1 and interleukin 1 receptor-like 1 [194, 206–208]. Thus, activation of ALK1 by TGF-β functions preferentially in regulating angiogenesis activation. Interestingly, the decision by TGF-β to couple to either ALK1 or ALK5 likely depends on the balance between TGF-β and

angiogenic cytokines within tumor microenvironments. Indeed, low TGF-β concentrations enhance the ability of bFGF and VEGF to stimulate EC proliferation and angiogenic sprouting, while high TGF-β concentrations inhibit these angiogenic activities [185, 186].

In addition to regulating angiogenesis *via* its activation of ALK1 and ALK5, TGF-β also controls vessel development *via* its actions on the co-receptors, TβR-III/betaglycan and endoglin. Several recent studies have shown the importance of epithelial cell expression of TβR-III in suppressing the growth and metastasis of breast [81], lung [82], pancreatic [83], ovarian [84], and prostate [85]cancers. However, the ability of soluble TβR-III to bind and sequester TGF-β has been used successfully to antagonize tumor angiogenesis and, consequently, to inhibit the growth and progression of human tumors produced in mice [209–211]. In contrast, endoglin is expressed predominantly on proliferating ECs, and its expression also can be upregulated by ALK1 [208]. When expressed in human umbilical vein endothelial cells, endoglin inhibits ALK5 signaling and promotes EC proliferation, migration, and tubulogenesis in conjunction with stimulation of ALK1 [212, 213]. Moreover, the redundant clinical symptoms of HHT1 (*i.e.*, endoglin defects) and HHT2 (*i.e.*, ALK1 defects) in humans, together with the overlapping phenotypes observed between ALK1- and endoglin-deficient mice, suggest that ALK1 and endoglin both function as negative regulators of TGF-β/ALK5 signaling.

Collectively, these studies highlight the complexities associated with the ability of TGF-β to regulate EC activities coupled to angiogenesis. Future studies clearly need to *(i)* better define the precise mechanisms that enable TGF-β and its downstream effectors to govern the induction of angiogenic or angiostatic gene expression profiles; *(ii)* establish the impact of EC and perivascular cell differentiation states to influence the angiogenic response to TGF-β; and *(iii)* identify the microenvironmental cues and signals the cooperate with TGF-β in mediating angiogenesis activation and resolution.

6. TGF-β and cancer cell survival

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Although cancer typically is viewed as a disease that arises from uncontrolled cell proliferation, it also is a disease of dysregulated apoptosis that enhances cancer cell survival by conferring developing neoplasms resistance to stimuli that would normally induce their programmed cell death [2]. Under normal physiological conditions, TGF-β inhibits autonomous cell growth by inducing cell cycle arrest or by stimulating cellular differentiation [3, 4, 214]; however, TGF-β also guards against the development of dysregulated cell growth through its ability to promote programmed cell death, particularly in lymphocytes and hepatocytes [3, 4, 214, 215]. Importantly, TGF-β stimulation of apoptosis occurs independently of its regulation of EMT and cell motility [216, 217], and is mediated through its regulation of a variety of proapoptotic proteins (Table 1).

Table 1

Expression of Proapoptotic Genes Induced by TGF-β

One of the hallmarks of cancer is the ability of malignant cells to acquire resistance to apoptotic stimuli [2]. As mentioned previously, tumorigenesis frequently subverts the tumor suppressing activity of TGF-β, thereby enabling TGF-β to promote oncogenesis by stimulating the growth, invasion, metastasis, and angiogenesis of developing tumors (*see above*). Along these lines, alterations within the TGF-β signaling system also conspire to convert TGF-β from activator of apoptosis to a promoter of cancer cell survival. This altered cellular response to TGF-β is especially evident through its ability to protect and promote the recovery of cancer cells following their targeting by radiation and chemotherapy treatments [218, 219]. Thus, the continued reliance on radiation and chemotherapy regimens to treat cancer patients necessitates that science and medicine fully elucidate the molecular mechanisms that affords TGF-β to protect developing neoplasms from apoptotic stimuli.

Numerous studies have established that dysregulated activation of the NF-κB and PI3K/AKT pathways by TGF -β functions in promoting cancer cell survival during tumorigenesis [220–223]. Interestingly, TGF-β routinely

suppresses the activation of NF-κB in normal epithelial cells by inducing their expression of IκBα, a phenomenon that is inactivated during oncogenic progression [157, 215]. Quite intriguingly, TGF-β stimulation of late stage cancer of the breast, prostate, and liver results in NF-κB activation, leading to the induction of pro -survival and -tumorigenic gene expression profiles [220, 224–226]. The aberrant activation of NF-κB by TGF-β has been attributed its stimulation of TAK1, which induces IKK activation [220, 224, 225]. Indeed, recent work in our laboratory defined a novel TAB1:TAK1:IKKβ:NF-κB signaling axis that forms aberrantly in breast cancer cells, and in normal mammary epithelial cells following their induction of EMT by TGF-β [225]. Once formed, this signaling axis enables oncogenic signaling by TGF-β in part *via* activation of NF-κB and its consequential production of proinflammatory cytokines, and of Cox-2 [227], which engages a PGE2:EP2 signaling axis coupled to breast cancer development and progression [228]. In addition, TAK1-deficiency abrogated the ability of TGF-β to induce the invasion of breast cancer cells, as well as restored their ability to undergo cytostasis in response to TGF-β. More importantly, expression of a dominant-negative TAB1 mutant dramatically reduced the growth of mammary tumors in normal mice, as well as in their immunocompromised counterparts, suggesting a potentially important role of NF-κB in regulating innate immunity by TGF-β [225]. Recently, TGF-β was shown to induce xIAP expression in hepatic and endometrial carcinomas, which enhanced their survival and invasiveness *via* xIAP-mediated regulation of PK3K/AKT, TAK1, PKC, JNK, and MMP-9 [229, 230]. Along these lines, work in our laboratory has identified xIAP as an essential mediator underlying the formation of TAB1:TAK1:IKKβ complexes and, consequently, their activation of NF-κB solely in cancer cells [231]. Thus, pharmacological targeting of xIAP may represent a novel approach to antagonize the oncogenic activities of TGF-β in developing and progressing human malignancies.

Altered coupling of TGF-β that results in its stimulation of AKT [232] and p38 MAPK [233] may also serve in potentiating the activation of IKKβ and NF-κB. Furthermore, activated IKK [234] complexes and AKT [223] both directly phosphorylate FoxO, which subsequently is inactivated and sequestered in the cytoplasm by its binding to 14-3-3 proteins. In doing so, phosphorylated FoxO transcription factors are no longer available to participate in Smad2/3-mediated expression of apoptotic and cytostatic genes, thereby enhancing cancer cell survival. Finally, activated AKT has been shown to bind and sequester inactive, cytoplasmic Smad3, which prevents TGF-β stimulation of programmed cell death [110, 111].

In addition to its anti-apoptotic function, NF-κB also plays an important role in mediating TGF-β stimulation of EMT and metastasis [168], and of pro-inflammatory gene expression [226]. For instance, inhibiting NF-κB activity in Ras-transformed mammary epithelial cells blocked the ability of TGF-β to *(i)* induce MMP-13 and MCP1 expression, and *(ii)* repress E-cadherin expression. Moreover, TGF-β stimulation of EMT is sufficient in eliciting its ability to stimulate, not inhibit, NF-κB activity in mammary epithelial cells [225]. When stimulated with TGF-β, cancer cells synthesize and secrete an array of pro-inflammatory genes following NF-κB activation. Included in this list of proinflammatory mediators regulated by TGF-β and its activation of NF-κB are Cox-2, GM-CSF, TNF-α, and interleukins 1, 6, and 8 [225, 226, 235, 236], which collectively drive oncogenesis by stimulating tumor angiogenesis and metastasis, as well as by inhibiting host immunosurveillance. Recently, aberrant expression of Cox-2 was shown to negate the cytostatic activities of TGF-β [237], and to promote its ability to induce breast cancer metastasis to bone [235]. Thus, pharmacological targeting of Cox-2 may enhance the tumor suppressing activities of TGF-β. Finally, TGF-β also enhances cancer cell survival through its ability to modulate and suppress immunosurveillance mediated by the adaptive immune system [238, 239]. The role of TGF-β in regulating immune system function during tumorigenesis is discussed below.

7. TGF-β and dysregulated immunosurveillance

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It has been appreciated from many years now that cancer initiation, promotion, and progression all are linked to aberrant and/or persistent inflammation within tumor microenvironments [240]. Indeed, the balance between host immunosurveillance and proinflammatory activity within the tumor milieu plays an essential role in determining whether tumor development and progression is induced or inhibited [240]. An implicit

factor in regulating this delicate balancing act is TGF-β, which governs the activities and behaviors of cancer cells and their associated stromal components [239]. Indeed, while many cytokines and chemokines participate in the inflammatory process, the prominent and essential role of TGF-β in maintaining proper immune system function and homeostasis is underscored by the finding that TGF-β1-deficient mice exhibit lethal multifocal inflammatory disease [241, 242], while mice lacking Smad3 exhibit defects in the responsiveness and chemotaxis of their neutrophils, and their T and B cells [243]. In addition, a characteristic feature of cancer cells is their capacity to increase the production and secretion of TGF-β into tumor microenvironments, and into the general circulation of cancer patients [244–246]. Elevated concentrations of active TGF-β also are detected within the tumor milieu due to enhanced ECM degradation mediated by resident and recruited leukocytes –*i.e.*, monocytes/macrophages, dendritic cells, granulocytes, mast cells, T cells, and natural killer (NK) cells – that either promote or suppress tumor development in a context-specific manner [240]. It is interesting to note that the recruitment of leukocytes to developing neoplasms in many respects reflects the processes underlying their normal recruitment to regions of wounding, inflammation, or infection. This observation, coupled with the fact that cancer cells house genetic or epigenetic alterations that elicit persistent tumor inflammation and chronic leukocyte infiltration undoubtedly led to Dvorak to liken "tumors to wounds that never heal" [247].

In general, high levels of TGF-β inactivate host anti-tumor immunosurveillance systems, which confers immune privilege to developing neoplasms and ensures for their continued progression. Mechanistically, TGF-β inhibits the proliferation and differentiation of NK and T cells, as well as their production of cytotoxic effector molecules. Moreover, the ability of TGF-β to affect the behaviors of cytotoxic $CD8^+$ T cells occurs in a manner that reflects their differentiation status. For instance, TGF-β is a potent inhibitor of naïve $CD8^+$ T cell populations, but elicits little to no response in their fully differentiated and activated counterparts, which downregulate expression of TβR-II. The insensitivity of differentiated $CD8^+$ T cells to TGF-β can be overcome by the production of either IL-10 or IL-2, or by the expression of the co-stimulatory molecule CD28 [238, 239, 248]. Interestingly, TGF-β stimulation of T cells expressing CD28 ultimately promotes the survival of memory/effector phenotypes in thymic and peripheral T cell populations.

The lymphocyte defects observed in Smad3-deficient mice suggest an important role for this latent transcription factor in mediating immunosuppression by TGF-β. Accordingly, activation of Smad3 by TGF-β prevents mitogenesis in $CD8^+$ T cells by *(i)* inhibiting their production of IL-2; *(ii)* repressing their expression of c-Myc, cyclin D2, and cyclin E; and *(iii)* stimulating the expression of the CDKIs p15, p21, and p27 [239, 248, 249]. In addition, TGF-β also represses the expression and production of cytotoxic effector molecules, including IFN-γ, lymphotoxin-α (LT- α), perforin/granzyme, and Fas ligand [239, 248, 249]. Although TGF-β has no effect on proliferation of $CD4^+$ T cells, it does inhibit $CD4^+$ T cell differentiation into T helper 1(Th1) and Th2 cell lineages, which readily secrete IFN-γ and LT-α (Th1) or IL-4, IL-5, and IL-13 (Th2), respectively. The inhibitory activities of TGF-β in $CD4^+$ T cells result from its ability to downregulate T cell receptor expression, to diminish intracellular Ca^{++} signaling, and to reduce the expression and activation of transcription factors [239, 248, 249], which collectively serve to alleviate host immunosurveillance. Accordingly, inactivation of TGF-β in $CD8^+$ or $CD4^+$ T cells results in T cell-mediated eradication of skin [250] and prostate [251] in mice. The ability of TGF-β to suppress the activities of tumor-infiltrating T cells also has been associated with its activation of Tregs, which represent a subset of CD4+ and CD8+ peripheral T cells that also express CD25 and Fox3P, and which routinely localize to tumor microenvironments where they promote immune privilege by inhibiting tumor targeting T and NK cells [248].

In addition to regulating the adaptive immune system, TGF-β also figures prominently in governing the behavior and activation of the innate immune system. Indeed, TGF-β is a potent inhibitor of NK cell cytolytic activity, presumably by attenuating NKp30 and NKG2D receptor activation. Neutralizing TGF-β not only prevents NKG2D downregulation, but also restores NK cell anti-tumor reactivity [252]. In addition, NK cell production of IFN-γ is repressed by TGF-β through cellular mechanisms that remain incompletely understood. Dendritic cells are professional antigen presenting cells whose expression of MHC class II, of the co-

stimulatory molecules CD40, CD80, and CD86, and of cytokines TNF-α, IL-12, and CCL5/Rantes is inactivated following their stimulation with TGF-β [239, 248, 249, 253]. In addition, the increased production of TGF-β within tumor microenvironments serves as a chemoattractant for mast cells that enhance tumor progression through their synthesis and release of a variety of factors, including histamine, proteases, and cytokines (*e.g.*, VEGF and TGF-β) [249, 254]. Lastly, TGF-β stimulation of resting monocytes (*i.e.*, non-phagocytic) promotes their chemotaxis to and infiltration into tumor microenvironments where they *(i)* enhance oncogenesis by stimulating ECM degradation, and by inducing tumor angiogenesis, invasion, and metastasis, and *(ii)* contribute to immunosuppression through upregulated production and release of TGF-β into tumor microenvironments [255, 256]. Furthermore, activation of differentiated macrophages by TGF-β inhibits their expression of the scavenger receptors, CD36 and SR-A, of the opsonizing IgG receptors, FcγRI and FcγRIII, and of the antigen presenting receptor, MHC class II, which collectively function to enhance the progression and survival of developing neoplasms. In addition to macrophages, TGF-β also induces the infiltration of tumor-associated neutrophils (TAN) that bear a tumor promoting phenotype, and as such, antagonizing TGF-β function facilitates the recruitment of TANs that possess an anti-tumor phenotype [257].

8. TGF-β and cancer cell immortality

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An important and widely recognized hallmark of cancer lies in the ability of malignant cells to become immortal, a process achieved through the reactivation of human telomerase reverse transcriptase (hTERT) and its consequential lengthening of chromosomal telomeres. Structurally, telomeres are complex nucleoproteins comprised of single- and double-stranded DNA and associated proteins that function in safeguarding against chromosomal end-to-end fusions. With the exception of germ cells, hTERT expression is silenced and undetectable in normal somatic cells. Thus, in the absence of hTERT and its ability to maintain telomere integrity, each successive cell division results in telomere erosion and, ultimately, in cell senescence, crisis, and apoptosis [258]. Collectively, these events conspire to suppress dysregulated cell growth and survival, and as such, to prevent tumor formation.

In contrast to their normal counterparts, cancer cells readily overcome the negative constraints that repress hTERT expression, resulting in the reinitiation and activation of telomerase activity and the acquisition of cellular immortalization. Human telomerase consists of a telomerase RNA template (hTER) and hTERT, both of which function in coordinating the synthesis of telomere repeats that mediate chromosomal integrity and prolong cellular replication [258, 259]. Given the importance of dysregulated hTERT expression and activity to promoting cancer development, it is fitting that TGF-β may in fact be the principle cytokine operant in silencing hTERT expression in normal cells [260]. Indeed, the extent of TGF-β signaling in responsive cells displays an inverse relationship with the levels of hTERT expression observed in cancers of the colon and breast [260–263]. Moreover, elevating hTERT expression in epithelial cells elicits resistance to the cytostatic activities of TGF-β [264].

Current evidence indicates that TGF-β represses hTERT expression *via* several distinct mechanisms. For instance, cellular depletion of SIP1/ZEB2, a zinc-finger transcription factor more commonly associated with induction of EMT, significantly attenuates the ability of TGF-β to repress hTERT expression in human osteosarcoma cells [265]. Moreover as mentioned above, TAK1 plays a major role in promoting the activation of NF-κB by TGF-β in breast cancer cells, thereby enhancing their tumorigenicity in mice [225]. Quite surprisingly, activation of TAK1 by TGF-β also has been linked to the repression of hTERT expression in human lung cancer cells. In doing so, TAK1 suppresses Sp1 transcriptional activity *via* recruitment of HDAC to the hTERT promoter [266]. Future studies clearly are warranted to delineate how TAK1 participates in mediating tumor suppression (*i.e.*, repressing hTERT expression) and tumor promotion (*i.e.*, NF-κB activation and proinflammatory gene expression) in response to TGF-β. Along these lines, upregulated expression of the negative regulator of TGF-β signaling, Smurf2, contradicts the immortalization activities of hTERT in a manner independent of the ability of Smurf2 to ubiquitinate target proteins and inhibit TGF-β signaling [267]. Thus, the interplay between Smurf2, hTERT, and TGF-β awaits further clarification. Finally, two recent studies

have shown that TGF-β represses hTERT transcription by inducing the assembly of Myc:Smad3 complexes on E-box and SBE sites in the hTERT promoter [262]. Thus, dysregulated Myc expression or aberrant Smad3 signaling can promote upregulated telomerase expression and activity.

9. Concluding remarks

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Defining the molecular mechanisms that underlie the initiation of the TGF-β paradox in human malignancies remains the most important and unanswered question concerning the biology and pathology of this multifunctional cytokine. In their seminal paper, Hanahan and Weinberg [2] proposed that all neoplasms must evolve 6 physiological traits during the course of their malignant progression. Importantly, the acquisition of each trait – *i.e.*, autonomous cell growth, resistance to cytostatic, apoptotic, and morality signals, and the induction of angiogenesis and invasion/metastasis – represents an essential rate-limiting step operant in mediating cancer development and progression. The studies reviewed herein show that TGF-β plays a major role, both directly and indirectly, in regulating the acquisition by cancer cells of each of these cancer hallmarks, and as such, the development of novel TGF-β chemotherapeutics capable of targeting these cancer hallmarks offers new inroads into alleviating the devastating effects of TGF-β in promoting metastatic disease development in humans.

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Footnotes

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The Cain and Abl of Epithelial-Mesenchymal Transition and Transforming Growth Factor-β in Mammary Epithelial Cells

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Key Words

Breast cancer · c-Abl · Epithelial-mesenchymal transition · Metastasis · Signal transduction · Transforming growth factor-β

Abstract

Transforming growth factor-β (TGF-β) normally inhibits breast cancer development by preventing mammary epithelial cell (MEC) proliferation, by inducing MEC apoptosis, and by creating cell microenvironments that maintain MEC homeostasis and prevent their uncontrolled growth and motility. Mammary tumorigenesis elicits dramatic alterations in MEC architecture and microenvironment integrity, which collectively counteract the tumor-suppressing activities of TGF-β and enable its stimulation of breast cancer invasion and metastasis. How malignant MECs overcome the cytostatic actions imposed by normal microenvironments and TGF-β, and how abnormal microenvironments conspire with TGF-β to stimulate the development and progression of mammary tumors remains largely undefined. These knowledge gaps have prevented science and medicine from implementing treatments effective in simultaneously targeting abnormal cellular microenvironments, and in antagonizing the oncogenic activities of TGF-β in developing and progressing breast cancers. c-Abl is a ubiquitously expressed nonreceptor protein tyrosine kinase that essentially oversees all aspects of cell physiology, including the regulation of cell proliferation, migration and adhesion, as well as that of cell survival. Thus, the biological functions of c-Abl are highly reminiscent of those attributed to TGF-β, including the ability to function as either a suppressor or promoter of tumorigenesis. Interestingly, while dysregulated Abl activity clearly promotes tumorigenesis in hematopoietic cells, an analogous role for c-Abl in regulating solid tumor development, including those of the breast, remains controversial. Here, we review the functions of c-Abl in regulating breast cancer development and progression, and in alleviating the oncogenic activities of TGF-β and its stimulation of epithelial-mesenchymal transition during mammary tumorigenesis.

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Abbreviations used in this paper

BCR	break-point cluster region
CML	chronic myelogenous leukemia
CST-Abl	constitutively active c-Abl
EMT	epithelial-mesenchymal transition
MEC	mammary epithelial cell
PTK	protein tyrosine kinase
TGF-β	transforming growth factor-β

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Introduction

Transforming growth factor-β (TGF-β) is a ubiquitous cytokine that fulfills fundamental roles during embryonic development, cellular differentiation, wound healing and tissue remodeling, as well as immune homeostasis [Massague, 2008; Heldin et al., 2009; Tian and Schiemann, 2009b]. In addition, TGF-β also plays an essential function in maintaining normal epithelial cell and tissue architecture, a regulatory mechanism that becomes disrupted in developing neoplasms. Indeed, as neoplastic lesions progress and become invasive, they typically circumvent the tumor-suppressing activities of TGF-β and paradoxically convert this cytokine into a potent promoter of metastatic dissemination [Benson, 2004; Buck and Knabbe, 2006; Pardali and Moustakas, 2007; Barcellos-Hoff and Akhurst, 2009; Wendt et al., 2009a]. Recent evidence has established epithelial-mesenchymal transition (EMT) as being a vital component involved in initiating oncogenic TGF-β signaling in normal and malignant cells [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Indeed, TGF-β is a master regulator of EMT and its ability to engender polarized epithelial cells to (1) downregulate their expression of genes associated with epithelial phenotypes, including those operant in forming adherens and tight junctions; (2) remodel their actin cytoskeletons and microtubule networks; and (3) upregulate their expression of genes associated with mesenchymal phenotypes and cell motility [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. The process of EMT has recently been categorized into 3 distinct biological subtypes [Kalluri and Weinberg, 2009], namely type 1 (embryonic and developmental EMT), type 2 (tissue regeneration and fibrotic EMT) and type 3 (cancer progression and metastatic EMT). The linkage of type 3 EMT to the development of metastasis and poor clinical outcomes [Thiery, 2003] has led to intense research efforts aimed at developing novel chemotherapeutics capable of inhibiting oncogenic EMT, and as such, of improving the clinical course of patients with metastatic disease. Alternatively, identifying the molecular mechanisms that promote mesenchymal-epithelial transition (MET), which phenotypically and morphologically reverses the activities of EMT, may also offer new inroads to impede or thwart primary tumor metastasis, an idea echoed by those who attended the 3rd International TEMTIA meeting that was held in Krakow, Poland, in 2007.

c-Abl is a multifunctional nonreceptor protein tyrosine kinase (PTK) that localizes to the plasma membrane, cytoplasm and nucleus where it governs a variety of cellular functions and activities, including the (1) transduction of integrins and growth factor receptor signals; (2) induction of cell cycle arrest initiated by DNA damage; (3) regulation of actin cytoskeletal dynamics; and (4) interaction with numerous adaptor proteins and scaffold complexes [Pendergast, 1996; Plattner et al., 1999; Hamer et al., 2001; Woodring et al., 2001; Pendergast, 2002; Woodring et al., 2002; Zandy and Pendergast, 2008]. In addition, c-Abl and its relative Arg are unique among nonreceptor PTKs in that both molecules house direct actin-binding domains that enable c-Abl to sense and respond to extracellular signals coupled to altered actin cytoskeletal architectures [Woodring et al., 2001, 2002; Zandy and Pendergast, 2008]. It is interesting to note that the diverse and complex biological functions of c-Abl are surprisingly reminiscent of the pathophysiological actions of TGF-β, including its dichotomous behavior exhibited during tumorigenesis. For instance, the tumor-promoting activities of c-Abl are best exemplified by its causal initiation of chronic myelogenous leukemia (CML), wherein c-Abl is translocated and fused to the break-point cluster region (BCR) on chromosome 22, resulting in the generation of a constitutively active Abl kinase fusion protein [Druker, 2006; Wang, 2006; Hunter, 2007; Lin and Arlinghaus, 2008]. Moreover, the pharmacological development and clinical implementation of imatinib (also known as Gleevec or STI-571), which targets the ATP-binding site in the c-Abl kinase domain and inhibits its phosphotransferase activity [Druker, 2006; Wang, 2006; Hunter, 2007; Lin and Arlinghaus, 2008], has significantly improved the treatment of CML and served as a model for the rationale design of protein kinase inhibitors [Druker, 2006; Soverini et al., 2008]. Although dysregulated c-Abl activity clearly promotes tumorigenesis in hematopoietic cells, the role of c-Abl in regulating tumorigenesis in solid tumors remains controversial. In fact, recent clinical trials designed to assess the efficacy of c-Abl antagonism in preventing breast cancer progression failed to observe any clinical benefit in imatinib-treated breast cancer patients. Moreover, these same studies found imatinib to cause significant toxicity and elicit disease progression in breast cancer patients [Modi et al., 2005; Chew et al., 2008; Cristofanilli et al., 2008]. Along these lines, our recently published study showed that imatinib administration failed to provide any therapeutic benefit to mice bearing aggressive mammary tumors, and instead, this same pharmacological treatment tended to produce larger breast tumors as compared with those observed in their vehicle-treated counterparts (fig. 1) [Allington et al., 2009]. Remarkably,

this same study demonstrated that engineering late-stage metastatic breast cancer cells to stably express a constitutively active c-Abl mutant resulted in their morphologic and phenotypic reversion both in vitro and in vivo, as well as circumvented the oncogenic activities of TGF-β and its ability to induce EMT [Allington et al., 2009]. Clearly, a deeper and more thorough understanding of pathophysiological functions of c-Abl in regulating solid tumor development and progression is needed for science and medicine to successfully advance the use of targeted chemotherapies against c-Abl and against various effectors activated by oncogenic TGF-β signaling.

Here, we review recent findings that directly impact our understanding of the dichotomous roles played by c-Abl during mammary tumorigenesis and discuss emerging evidence suggesting that chemotherapeutic targeting of c-Abl activation, not inhibition, may offer new inroads to suppress breast cancer progression and the oncogenic activities of TGF-β, particularly its induction of EMT and metastasis in developing neoplasms of the breast.

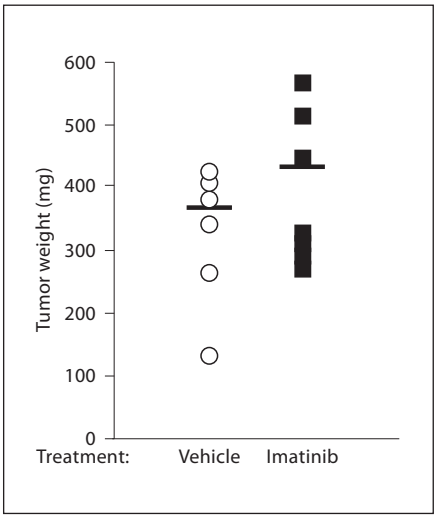

Fig. 1. c-Abl antagonism fails to inhibit mammary tumor growth in mice. Female Balb/c mice were injected orthotopically with syngeneic 4T1 cells and treated daily beginning on day 8 after engraftment with either vehicle or imatinib (50 mg/kg/day) as indicated. Primary tumors were removed surgically and weighed at days 24 and 27 after engraftment. Bars indicate the mean tumor weight per group (6 mice/group). Reprinted with kind permission from Allington et al. [2009].

Abl Signaling and Cancer Progression

c-Abl was originally identified as the cellular counterpart to the Abelson murine leukemia virus, v-Abl [Wang et al., 1984]. Subsequent studies established that c-Abl exists in 2 isoforms in mammals (1a or 1b in humans and I or IV in mice), and that this large (approximately 140 kDa) nonreceptor PTK contains a variety of modular domains that enable c-Abl to bind numerous signaling and scaffolding proteins. Figure 2 depicts the structural features of human and murine c-Abl isoforms, both of which house (1) Src homology 2 (SH2) and SH3 domains; (2) a proline-rich adaptor-binding motif; (3) a PTK domain; (4) 3 nuclear localization signals and 1 nuclear export signal; (5) 3 high-mobility group-like boxes that function cooperatively in binding DNA; and (6) globular and filamentous actin-binding domains [Woodring et al., 2002; Hunter, 2007]. The presence of functional nuclear localization signals and nuclear export signal motifs localizes c-Abl to both the cytoplasmic and nuclear compartments in quiescent cells, as well as enables c-Abl to translocate to the nucleus in response to a variety of extracellular stimuli [Lewis et al., 1996; Wen et al., 1996; Taagepera et al., 1998; Plattner et al., 1999; Woodring et al., 2002]. In nontransformed cells, the activation status of c-Abl is tightly controlled and its PTK activity is retained in an inactive conformation through intramolecular c-Abl interactions [Woodring et al., 2002; Hunter, 2007]. Indeed, displacing either the N-terminal autoinhibitory Cap region or the SH2/SH3 domains of c-Abl by the binding of its substrates or effector molecules rapidly and transiently stimulates this PTK, whose activity is downregulated following its ubiquitination and proteosomal degradation [Echarri and Pendergast, 2001; Woodring et al., 2002; Zhu and Wang, 2004]. Cytoplasmic c-Abl is activated by a variety of growth factors and cytokines, by reactive oxygen species, and by cell attachment and mechanotransduction that directs c-Abl to adherens complexes and regions of actin cytoskeletal remodeling [Woodring et al., 2002; Zhu and Wang, 2004]. Nuclear c-Abl is also activated by reactive oxygen species and by DNA damage that couples c-Abl to the regulation of cell survival and apoptosis [Agami et al., 1999; Vigneri and Wang, 2001; Truong et al., 2003; Chau et al., 2004]. Interestingly, both of the oncogenic forms of c-Abl, namely BCR-Abl and v-Abl, are unable to enter the nucleus, and their enforced nuclear expression induces apoptosis, not cellular transformation [Woodring et al., 2002; Zhu and Wang, 2004; Suzuki and Shishido, 2007]. Thus, the pathophysiological output of c-Abl activation ultimately reflects a conglomeration of the initiating signal, the cellular context and the cellular locale wherein c-Abl is stimulated.

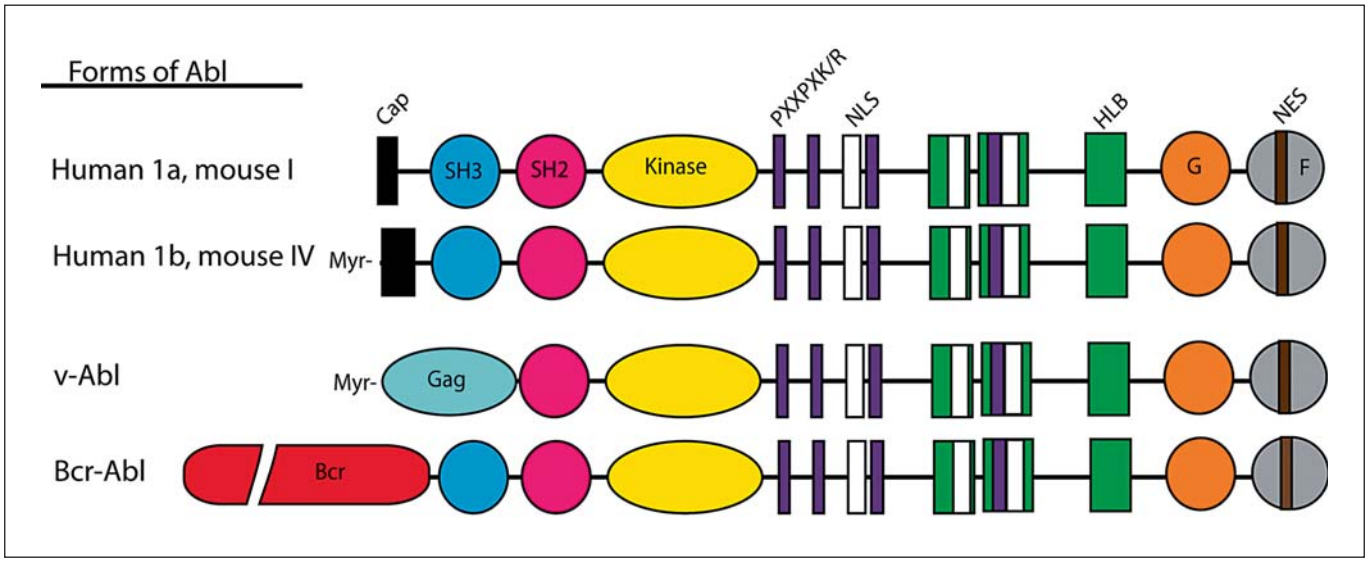

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Fig. 2. Schematic depiction of the functional domains of human (1a and 1b) and murine (I and IV) Abl isoforms. The N terminus of c-Abl contains either an autoinhibitory Cap region or a consensus motif for myristoylation (black), which is followed by SH3 (blue; colors refer to the online version only) and SH2 (pink) domains, which is followed by the catalytic PTK domain (kinase, yellow). The central region of c-Abl possesses 4 PXXPXK/R sequences (purple), 3 nuclear localization sequences (NLS, white), and 3 high-mobility group-like boxes (HLB, green). Finally, the C terminus of c-Abl houses domains for binding globular (G, orange) and filamentous (F, gray) actin, as well as a nuclear export sequence (NES, brown). The oncogenic forms of Abl (v-Abl and BCR-Abl) contain modified N-terminal regions that disrupt the autoinhibitory functions normally mediated by the Cap region, which elicits constitutive PTK activity. The aberrant N terminus in v-Abl comprises a viral Gag sequence (light blue), while that in BCR-Abl comprises a portion of the N terminus of the BCR (red).

As mentioned above, the oncogenic potential of c-Abl was elucidated by the discovery that c-Abl can be fused to the BCR region on chromosome 22, an untoward translocation event that gives rise to BCR-Abl and its ability to induce CML development and progression [Druker, 2006; Hunter, 2007]. The synthesis and implementation of imatinib (Gleevec) to antagonize the phosphotransferase activity of Abl revolutionized the treatment of CML by eliciting response rates of about 98% in patients with the chronic phase of CML at the time of diagnosis [Mauro and Druker, 2001; Mauro et al., 2002; Druker, 2006]. In stark contrast to its causal role in initiating hematopoietic cancers, a definitive function for c-Abl in promoting the formation and progression of solid tumors, including those of the breast, remains an active and controversial area of research. For instance, early cell biology studies found that the phosphorylation of Crk by c-Abl inhibits fibroblast and carcinoma cell motility by preventing the formation of Crk:p130Cas complexes [Kain and Klemke, 2001; Kain et al., 2003]. In addition, hepatocyte growth factor signaling and its stimulation of thyroid cancer cell migration was potentiated significantly in imatinib-treated cells as compared with their vehicle-treated counterparts [Frasca et al., 2001], suggesting that c-Abl activity suppresses carcinoma motility. With respect to cancers of the breast, c-Abl activation has been associated with enhanced breast cancer cell proliferation, invasion, survival and anchorage-independent growth [Plattner et al., 1999; Srinivasan and Plattner, 2006; Lin and Arlinghaus, 2008; Srinivasan et al., 2008], and with their transformation by Src [Sirvent et al., 2007]. In stark contrast, c-Abl was observed to be essential in suppressing mammary tumorigenesis mediated by ephrin B2/ephrin B [Noren et al., 2006]. Moreover, we recently discovered that constitutive c-Abl activity abrogates the oncogenic behaviors of TGF-β in late-stage breast cancer cells, resulting in their phenotypic and morphologic reversion both in vitro and in vivo (see below) [Allington et al., 2009]. These latter findings suggest that imatinib administration may be contraindicated in breast cancer patients. Accordingly, recent clinical trials designed to assess the efficacy of c-Abl antagonism in preventing breast cancer progression have met with disappointing results, including the presence of severe drug

Table 1. Role of c-Abl in solid tumor progression

Study type	Disease/cells	Major finding	Reference
Abl as a tumor promoter			
Preclinical	lung cancer	increased c-Crk expression is associated with aggressive phenotypes	Miller et al., 2003
Preclinical	lung cancer	imatinib and Fus1 inhibit c-Abl and anchorage-independent growth of H1299 cells	Lin et al., 2007b
Preclinical	breast cancer	constitutive c-Abl activation is elicited by dysregulated EGFR, HER2 and Src, leading to increased cell invasion	Srinivasan and Plattner 2006
Preclinical	breast cancer	c-Abl mediates Src-transformation and survival of breast cancer cells	Sirvent et al., 2007
Preclinical	breast cancer	c-Abl mediates IGF-1 receptor-stimulated breast cancer progression	Srinivasan et al., 2008
Abl as a tumor suppressor			
Preclinical	colon cancer	c-Abl activates p73α/GADD45α, leading to apoptosis in response to DNA mismatch repair	Li et al., 2008
Preclinical	colon cancer	c-Abl activates p73α/GADD45α, leading to G2 arrest after induction of DNA mismatch repair	Wagner et al., 2008
Preclinical	breast cancer	ephrin B2/ephrin B4 suppress breast cancer tumorigenicity via activation of a c-Abl/Crk/MMP-2-signaling axis	Noren et al., 2006
Preclinical	thyroid cancer	imatinib enhances thyroid cancer cell motility in response to HGF	Frasca et al., 2001
Preclinical	breast cancer	activated c-Abl suppresses oncogenic TGF-β signaling, inhibits EMT and reverts breast cancer tumorigenicity in vitro and in vivo	Allington et al., 2009
Clinical phase I	breast cancer	imatinib offered no clinical benefit in PDGF receptor-positive metatastic breast cancer	Cristofanilli et al., 2008
Clinical phase II	breast cancer	imatinib provided no therapeutic benefit against invasive breast cancer patients	Modi et al., 2005
Clinical phase II	breast cancer	imatinib and capecitabine treatment failed to improve the clinical course of metastatic breast cancer patients	Chew et al., 2008
Clinical phase I/II	prostate cancer	imatinib administration either alone or in combination promoted disease progression and severe toxicity	Lin et al., 2006, 2007a
Clinical phase II	pancreatic cancer	imatinib administration fails to offer any therapeutic protection against pancreatic cancer	Chen et al., 2006; Gharibo et al., 2008

EGFR = Epidermal growth factor receptor; HER2 = human epidermal growth factor receptor 2; IGF-1 = insulin growth factor 1; MMP-2 = matrix metalloproteinase 2; HGF = hepatocyte growth factor; PDGF = platelet-derived growth factor.

toxicities and the initiation of disease progression [Modi et al., 2005; Chew et al., 2008; Cristofanilli et al., 2008]. Similar detrimental clinical outcomes were observed in pancreatic [Chen et al., 2006; Gharibo et al., 2008] and prostate [Lin et al., 2006, 2007a] cancer patients subjected to imatinib administration.

Table 1 summarizes the dichotomous roles of c-Abl in regulating solid tumor development and progression, and in doing so, highlights the need to identify novel biomarkers capable of staging and stratifying cancer patients on the basis of their c-Abl expression and signaling profiles.

TGF-β Signaling and EMT

TGF-β is a pluripotent cytokine that plays essential roles in regulating mammalian development and differentiation, and in maintaining tissue homeostasis [Benson, 2004; Buck and Knabbe, 2006; Barcellos-Hoff and Akhurst, 2009; Tian and Schiemann, 2009b]. The versatile nature of TGF-β is emphasized by the fact that virtually all cells in the metazoan body are capable of both producing and responding to this cytokine. TGF-β is now recognized as a potent tumor suppressor that prevents the dysregulated growth and survival of cells, particularly those of epithelial, endothelial and hematopoietic origins [Massague, 2008; Heldin et al., 2009; Tian and Schiemann, 2009b]. The process of tumorigenesis and its associated genetic, epigenetic and microenvironmental alterations enable early-stage cancer cells to inactivate the cytostatic activities of TGF-β through mechanisms that remain incompletely understood. As these neoplastic cells continue to evolve towards advanced malignancy, they ultimately acquire the ability to convert the cytostatic functions of TGF-β into those capable of driving neoplastic progression, including the induction of tumor growth, invasion and metastatic dissemination [Benson, 2004; Buck and Knabbe, 2006; Barcellos-Hoff and Akhurst, 2009; Tian and Schiemann, 2009b]. The functional conversion

of TGF-β behavior during tumorigenesis is known as the 'TGF-β paradox', whose eventual interpretation and translation holds the key to developing novel chemotherapies capable of preferentially targeting the oncogenic activities of TGF-β [Schiemann, 2007].

An important consequence of TGF-β signaling is its potential to induce EMT, a process whereby immotile, polarized epithelial cells transdifferentiate into highly motile, apolar fibroblastoid-like cells [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Essential features exhibited by epithelial cells undergoing EMT include (1) diminished cell polarity owing to the downregulated expression of epithelial cell markers (e.g., E-cadherin, zona occludens 1 and β_4-integrin); (2) remodeled actin cytoskeletal architectures; (3) upregulated expression of fibroblast markers and genes operant in cell motility and invasion (e.g., vimentin, N-cadherin, α-smooth muscle actin, Twist); and (4) acquired expansion of cells that possess stem cell-like properties and phenotypes [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Importantly, recent studies by our group have shown that the initiation of oncogenic TGF-β signaling coincides with its stimulation of EMT in normal and malignant MECs [Galliher and Schiemann, 2006, 2007; Lee et al., 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b], suggesting that the eventual development and implementation of pharmacological agents capable of uncoupling TGF-β from EMT may one day improve the clinical course of breast cancer patients.

The ability of TGF-β to induce EMT commences upon its binding to the TGF-β type II receptor, which then recruits, transphosphorylates and activates TGF-β type I receptor, which then phosphorylates and stimulates the latent TGF-β transcription factors, Smad2 and Smad3. Following their activation, Smad2/3 interact physically with the co-Smad, Smad4, which enables the resulting heterocomplexes to translocate to the nucleus where they associate with a variety of transcriptional activators and repressors to govern the expression of TGF-β-responsive genes in a cell- and context-specific manner [Heldin et al., 2009; Wendt et al., 2009a]. The coupling of TGF-β to Smad2/3 stimulation, which is commonly referred to as either 'Smad2/3-dependent' or 'canonical' TGF-β signaling, plays an essential role in governing all aspects of the pathophysiological activities of TGF-β, including its induction of EMT [Oft et al., 1996; Valcourt et al., 2005; see also Masszi and Kapus, 2011, this issue]. In addition to its stimulation of canonical Smad2/3 signaling, we and others have identified a variety of noncanonical effectors whose activation by TGF-β also mediate fundamental functions of this cytokine [for review, see Lamouille and Derynck, 2011, this issue]. With respect to its induction of EMT, TGF-β must also activate (1) MAP kinase family members, particularly ERK1/2 [Xie et al., 2004] and p38 MAPK [Bhowmick et al., 2001; Galliher and Schiemann, 2007; Galliher-Beckley and Schiemann, 2008]; (2) focal adhesion complex proteins, including β_1- and β_3-integrins [Bhowmick et al., 2001; Galliher and Schiemann, 2006], Src [Galliher and Schiemann, 2006, 2007], focal adhesion kinase [Wendt and Schiemann, 2009] and p130Cas [Wendt et al., 2009b]; (3) nuclear factor-κB [Huber et al., 2004; Neil and Schiemann, 2008] and its downstream effector, cyclooxygenase 2 [Neil et al., 2008], which promotes EMT by initiating an autocrine prostaglandin E_2:EP_2 receptor signaling loop [Tian and Schiemann, 2009a]; (4) phosphoinositide 3-kinase and its downstream effectors, AKT and mTOR [Bakin et al., 2000; Lamouille and Derynck, 2007; Lamouille and Derynck, 2011]; (5) small guanosine triphosphate-binding proteins, including cdc42, Rac1 and RhoA [Wendt et al., 2009a]; and (6) PAR6 and its recruitment of the E3 ligase, Smurf1 [Ozdamar et al., 2005]. Although the precise contribution of canonical and noncanonical TGF-β signaling in mediating the various subtypes of EMT has yet to be clarified, it is known that the activation of both pathways is necessary for the faithful initiation and completion of EMT by TGF-β and its ability to confer stem cell-like properties to epithelial cells, whose newfound plasticity enables metastatic cancer cells to thrive in otherwise hostile secondary sites [Polyak and Weinberg, 2009]. Readers desiring more in-depth discussions of the molecular mechanisms that underlie the ability of TGF-β to induce EMT are directed to several recent and comprehensive reviews [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009; Lamouille and Derynck, 2011].

c-Abl Activation Suppresses EMT and Breast Cancer Progression

An essential component of EMT centers on the dissolution of adherens junctions and the remodeling of the actin cytoskeleton, both of which enable transitioning cells to acquire motile and invasive phenotypes [Heldin et al., 2009; Wendt et al., 2009a; Xu et al., 2009]. Interestingly, c-Abl activity has been associated with both the assembly and dissolution of adherens junctions and with altered actin dynamics and architectures through its

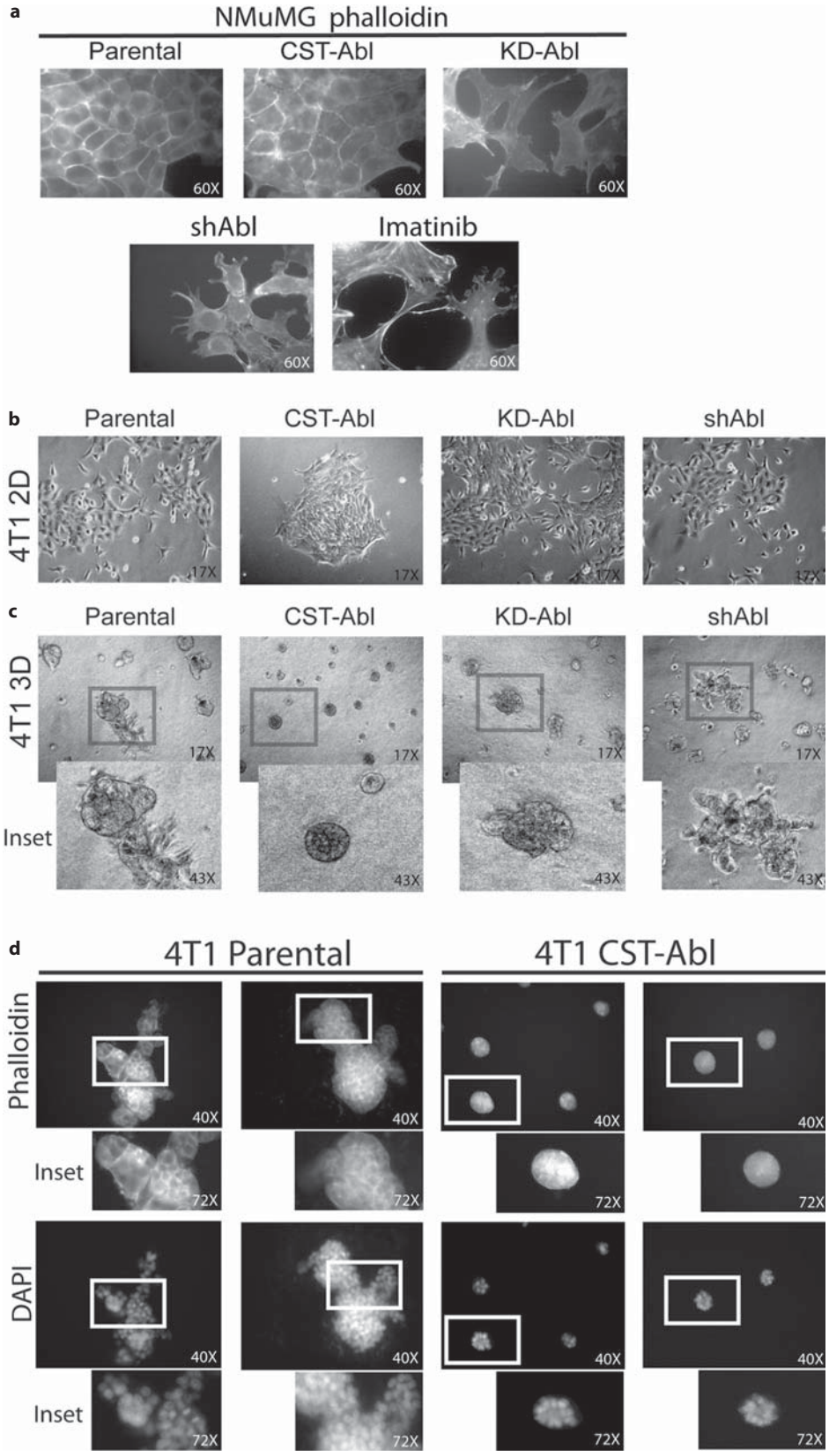
ability to bind globular and filamentous actin [Woodring et al., 2001, 2002, 2004; Zandy et al., 2007; Zandy and Pendergast, 2008]. Thus, the process of EMT presents a unique situation in which the paradoxical functions of c-Abl and TGF-β may intersect during mammary tumorigenesis. For instance, c-Abl essentially governs all biological decisions made by cells, including whether they proliferate, migrate or invade, or even whether they live or die [Zhu and Wang, 2004; Lin and Arlinghaus, 2008]. In fact, the physiological functions performed by c-Abl in many ways parallel those played by TGF-β, including their capacity to serve as tumor suppressors or promoters in a cell- and context-specific manner [Massague, 2008; Tian and Schiemann, 2009b]. Given the obvious pathophysiological similarities that exist between TGF-β and c-Abl in epithelial cells, we hypothesized c-Abl as an essential player in determining the morphologies and phenotypes of MECs, including their ability to undergo EMT in response to TGF-β. We tested this hypothesis by manipulating the expression or activity of c-Abl via several complementary approaches: (1) loss of c-Abl function by pharmacological inhibition (i.e. imatinib), by retroviral transduction of a kinase-dead c-Abl mutant or by lentiviral transduction of a shRNA against c-Abl; or (2) gain of c-Abl function by retroviral transduction of a constitutively active c-Abl mutant (CST-Abl). These c-Abl manipulations were applied to 2 murine MEC cell lines to interrogate the potential linkage between c-Abl and TGF-β: (1) normal murine NMuMG mammary gland cells, which are nontransformed and exhibit normal cuboidal epithelial architectures that readily undergo a robust EMT in response to TGF-β [Sokol et al., 2005; Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008; Lee et al., 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Wendt and Schiemann, 2009; Wendt et al., 2009b]; and (2) malignant, metastatic 4T1 cells, which are a late-stage model of TGF-β-responsive breast cancer [Galliher-Beckley and Schiemann, 2008; Lee et al., 2008; Neil and Schiemann, 2008; Yang et al., 2008; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b]. Figure 3a shows that c-Abl expression and activity were essential for maintaining normal MEC morphology, such that measures resulting in a loss of c-Abl function elicited an EMT response, while those measures resulting in a gain of c-Abl function produced a 'hyper-epithelial' response that was resistant to EMT and invasion stimulated by TGF-β [Allington et al., 2009]. The morphological alterations induced by inactivating c-Abl also transpired in normal human MECs [Allington et al., 2009], and more importantly, gene expression analyses confirmed the ability of c-Abl deficiency to increase mesenchymal gene expression (table 2). Thus, c-Abl inactivation results in morphological, transcriptional and migrational changes suggestive of those observed during EMT stimulated by TGF-β.

Table 2. c-Abl deficiency elicits an EMT transcriptional signature in NMuMG cells

NMuMG cells		Average relative mRNA, %	
		(–) TGF-β	(+) TGF-β
E-cadherin	scram	100.0	58.1 ± 8.7
	shAbl	104.6 ± 8.7	47.6 ± 7.8
N-cadherin	scram	100.0	856.0 ± 14.6
	shAbl	345.9 ± 21.1	1,050.9 ± 47.4
Vimentin	scram	100.0	475.6 ± 40.9
	shAbl	1,135.1 ± 26.9	858.0 ± 28.9
Twist	scram	100.0	295.3 ± 33.2
	shAbl	569.5 ± 20.3	683.9 ± 49.8

Quiescent parental (scrambled shRNA, scram) or c-Abl-deficient (shAbl) normal murine mammary gland cells (NMuMG) were incubated in the absence or presence of TGF-β_1 (5 ng/ml) for 48 h, at which point total RNA was isolated and subjected to semi-quantitative real-time PCR analysis. Data are the mean (±SE; n = 3) percent change in EMT marker gene expression relative to that observed in untreated parental normal murine mammary gland cells.

Extending these analyses to metastatic 4T1 cells demonstrated that CST-Abl expression was sufficient in reducing cell scattering and promoting stronger cell-cell junctions in traditional 2D culture systems (fig. 3b). The morphological differences induced by c-Abl activation were greatly exaggerated when these same cells were propagated in compliant 3D organotypic cultures. Indeed, in stark contrast to the large and irregularly shaped organoids formed by parental and loss of c-Abl function 4T1 cells, those expressing CST-Abl formed dramatically smaller and perfectly spherical organoids that appeared to undergo a partial hollowing (fig. 3c, d). In addition, CST-Abl expression reinstated the cytostatic activities of TGF-β in 4T1 cells in part by (1) acting as a broad-spectrum suppressor of matrix metalloproteinase expression [Allington et al., 2009], and (2) overriding the tumor-promoting signals engendered by rigid microenvironments [Allington et al., 2009]. Thus, enforced activation of c-Abl in metastatic MECs may provide a novel means to alleviate the oncogenic activities of TGF-β and, consequently,


a
NMuMG phalloidin
Parental
CST-Abl
KD-Abl
60X
shAbl
Imatinib
b
Parental
CST-Abl
KD-Abl
shAbl
4T1 2D
17X
c
4T1 3D
Inset
43X
d
4T1 Parental
4T1 CST-Abl
Phalloidin
DAPI
40X
72X

to phenotypically and morphologically normalize the malignant behaviors of breast cancer cells.

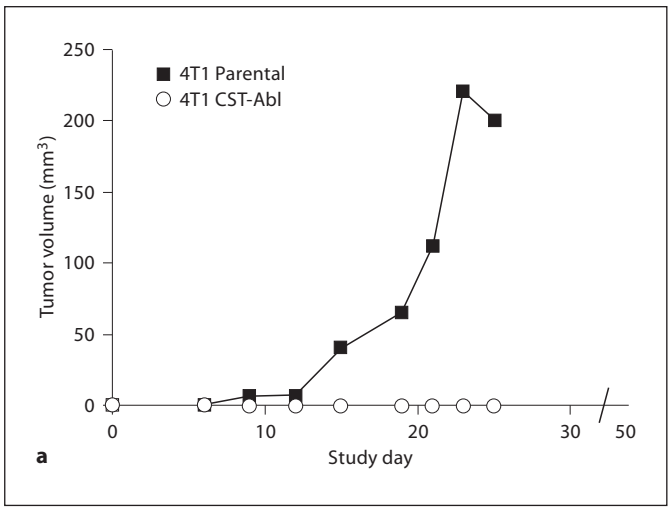
We tested the validity of the above supposition by orthotopically engrafting parental and CST-Abl-expressing 4T1 cells in syngeneic Balb/c mice. As expected [Galliher-Beckley and Schiemann, 2008; Neil and Schiemann, 2008; Wendt and Schiemann, 2009; Wendt et al., 2009b], parental 4T1 cells rapidly formed palpable tumors that necessitated host euthanization by day 28 due to excessive tumor burden (fig. 4a). Remarkably, every animal injected with CST-Abl-expressing 4T1 cells failed to develop palpable tumors during the course of the study (fig. 4a) and to exhibit overt signs of disease during necropsy [Allington et al., 2009]. Surprisingly, clonogenic assays facilitated the reisolation CST-Abl-expressing 4T1 cells from the mammary fat pads of mice that were euthanized on day 51 (fig. 4b). Collectively, these findings suggest that measures capable of enforcing c-Abl activation may represent a novel means to abrogate the oncogenic activities of TGF-β in cancers of the breast, and as such, to one day to improve the prognosis and treatment of patients with metastatic breast cancer. c-Abl may also influence the latency and dormancy of disseminated breast cancer in the form of micrometastases.

Chemotherapeutic Targeting of c-Abl in Breast Cancer: Friend or Foe?

Since the inception of the National Cancer Act of 1971, science and medicine have waged an all-out battle aimed at conquering cancer. Although considerable progress has been achieved in terms of our understanding of the molecular mechanisms that underlie neoplastic development and progression, cancer itself remains a significant health concern and burden in the United States. In fact, 1 in 4 deaths in the United States results from cancer, which is also the leading cause of death in individuals younger than 85 years of age [Jemal et al., 2009]. Despite these grim statistics, overall cancer incidence and mortality rates have begun to decline over the last decade, including those for breast cancer which annually contributes to more than 40,000 deaths and 192,000 new invasive cases of this deadly disease [Jemal et al., 2009]. Continuing along this positive trend will require the development of new diagnostic and chemotherapeutic regimens, as well as the elucidation of new knowledge of how cancer cells acquire the 6 essential phenotypes, or hallmarks, necessary to become malignant. Included in this phenotypic list is the ability of cancer cells to (1) disregard cytostatic signals; (2) grow autonomously; (3) stimulate angiogenesis; (4) ignore apoptotic signals; (5) become immortal; and (6) invade and metastasize [Hanahan and Weinberg, 2000]. The inability of developing neoplasms to acquire each of these phenotypes prevents their conversion to aggressive states, suggesting that these cancer hallmarks represent various rate-limiting steps during malignant development. Although EMT is not a recognized hallmark of tumorigenesis, type 3 EMT is essential for the acquisition of invasive and metastatic phenotypes by cancer cells and their development of chemoresistance. Thus, pharmacological targeting of individual cancer hallmarks and EMT, both singly and in combination, may offer new inroads to effectively treat the development and dissemination of human malignancies, particularly those of the breast.

Our findings showing that the c-Abl activation circumvents and overrides the oncogenic activities of TGF-β in normal and malignant MECs are intriguing in terms of their scientific and biological significance. For instance, alterations within tumor microenvironments can either restrain or free breast cancer progression in a manner that mirrors the conversion of TGF-β function from a suppressor to a promoter of tumor invasion and metastasis [Bierie and Moses, 2006]. Moreover, mounting evidence indicates that TGF-β promotes breast cancer progression in part via its reprogramming of MEC microenvironments and their cellular architectures. In attempting to circumvent the oncogenic activities of TGF-β in mammary tumors, science and medicine have employed a 'TGF-β centric' approach that is likely to fail

Fig. 3. Constitutive c-Abl activity suppresses EMT and induces MET in metastatic MECs. KD-Abl = Kinase-dead c-Abl mutant; shAbl = c-Abl deficient. **a** Direct FITC-conjugated phalloidin immunofluorescence was performed to monitor the actin cytoskeletal architecture in c-Abl-manipulated normal murine mammary gland (NMuMG) cells, which readily acquired mesenchymal morphologies in loss of c-Abl function MECs. **b** Bright-field images of c-Abl-manipulated 4T1 cells grown in traditional 2D tissue culture systems. Gain of c-Abl function elicited an apparent MET in 4T1 cells. **c**, **d** c-Abl-manipulated 4T1 cells were propagated for 7 days in compliant 3D organotypic cultures prior to analyzing their growth and morphology by capturing bright-field images (**c**), or by staining with FITC-conjugated phalloidin and DAPI (**d**). Gain of c-Abl function suppressed acinar growth and promoted normal acinar development, including partial hollowing of the resulting organoids. All are representative of 2–3 independent experiments and were reprinted with kind permission from Allington et al. [2009].



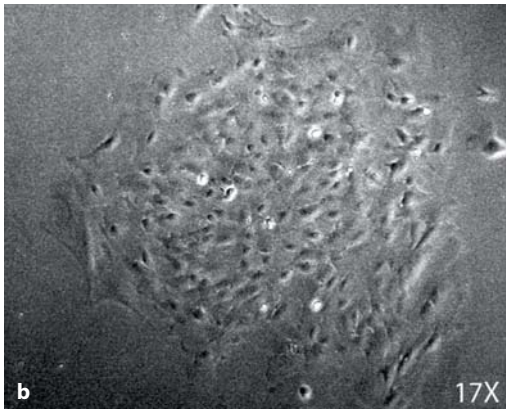


Fig. 4. Enforced c-Abl activation abrogates the growth of metastatic MECs in mice. **a** Female Balb/c mice were injected orthotopically with syngeneic parental (i.e. empty vector) or CST-Abl-expressing 4T1 cells (10,000 cells/animal; 12 animals/group), whose ability to grow as tumors was measured using digital calipers over a period of 30 days. Reprinted with kind permission from Allington et al. [2009]. **b** The mammary glands of mice injected with CST-Abl-expressing 4T1 cells were excised at day 51 after engraftment, and were subsequently dissociated enzymatically to produce a heterogeneous, single-cell suspension that was subjected to a clonogenic assay to reisolate reverted CST-Abl-expressing 4T1 cells.

clinically because targeted TGF-β therapies (i.e. both large and small molecule inhibitors) uniformly function as pan-TGF-β antagonists whose activities are subject to the phenomena underlying the 'TGF-β paradox' – i.e. the ability of mammary tumorigenesis to convert TGF-β from a tumor suppressor to a tumor promoter [Schiemann, 2007]. Pan-TGF-β antagonists are also inadequate in accounting for the pleiotropic functions of TGF-β in (1) governing MEC architectures and microenvironments, and (2) regulating tumor-associated stromal components. Thus, these findings underscore the necessity to design and implement rapid diagnostic tests capable of discriminating cancer patients most likely to benefit from targeted TGF-β therapies from those individuals most likely to be harmed by TGF-β antagonism.

A potential alternative to antagonizing all cellular responses to TGF-β may involve the implementation of a targeted approach that selectively inactivates specific noncanonical TGF-β effectors that preferentially promote its oncogenic activities. We have provided preclinical evidence that supports the therapeutic potential of this alternative approach (e.g., inactivation of αvβ3 integrin, Src, focal adhesion kinase, nuclear factor-κB, cyclooxygenase 2, or EP_2 receptor) [Galliher-Beckley and Schiemann, 2008; Neil and Schiemann, 2008; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b]; however, complete disease resolution has yet to be achieved using these applications due to their inability to phenotypically normalize and revert malignant MEC behaviors, architectures and microenvironments. Our findings demonstrate that the enforced activation of c-Abl can fulfill this latter requirement, and in doing so, can promote the phenotypic normalization and reversion of highly malignant, late-stage breast cancers in mice (fig. 4) [Allington et al., 2009]. To our knowledge, c-Abl activation represents the first bona fide tool competent to ablate the oncogenic activities of TGF-β, thereby restoring its cytostatic function in normalized and reverted MECs. Indeed, a bold extension of our findings leads us to propose that the development and implementation of allosteric c-Abl activators may one day provide a paradigm shifting the strategy to treat metastatic breast cancers. Clearly, the notion of chemically stimulating c-Abl is disconcerting to many scientists and clinicians, particularly since c-Abl has been linked to the oncogenic activities of the receptors for epidermal growth factor, platelet-derived growth factor and insulin-like growth factor 1, to the transforming activities of Src and signal transducer and activator of transcription 3, and to the prosurvival activities of ERK5 [Plattner et al., 1999; Srinivasan and Plattner, 2006; Sir-

vent et al., 2007; Lin and Arlinghaus, 2008; Srinivasan et al., 2008]. However, in all cases, it remains difficult to gauge the extent to which off-target effects of imatinib and other c-Abl inhibitors contribute to their apparent effectiveness against breast cancer cells in vitro. Moreover, data obtained in human clinical [Modi et al., 2005; Chen et al., 2006; Lin et al., 2006, 2007a; Chew et al., 2008; Cristofanilli et al., 2008; Gharibo et al., 2008] and murine preclinical trials (fig. 1) [Allington et al., 2009] clearly demonstrate the inability of imatinib to provide any chemotherapeutic benefit towards cancers of the breast. Along these lines, overexpression of c-Abl or the enforced nuclear expression of oncogenic Abl mutants (e.g., BCR-Abl or v-Abl) all fail to elicit cellular transformation [Zhu and Wang, 2004; Suzuki and Shishido, 2007], which points to the possibility that targeted c-Abl activation may in fact be well tolerated by normal MECs. Indeed, we find that MECs are exquisitely sensitive to subtle changes in c-Abl activity [Allington et al., 2009], and as such, we anticipate that even submaximal activation of c-Abl will prove sufficient to induce MET and suppress mammary tumorigenesis stimulated by TGF-β, thereby improving the clinical course of patients with metastatic breast cancer.

Unanswered Questions and Future Directions

Although our ability to accept or reject the aforementioned hypothesis clearly awaits additional experimentation in a variety of genetically distinct human breast cancer subtypes and tissues (e.g., luminal A vs. luminal B vs. ErbB2 vs. basal-like vs. normal-like) [Perou et al., 2000; Sorlie et al., 2001; Sorlie et al., 2003], our findings are nonetheless provocative and potentially paradigm changing with respect to redefining the role of c-Abl in regulating mammary gland development and neoplasia, and to establishing c-Abl as a novel suppressor of oncogenic TGF-β signaling in breast and other epithelial-derived cancers in part by inducing their phenotypic and morphologic normalization [Allington et al., 2009]. Figure 5 depicts our current understanding of the role of c-Abl in regulating normal and malignant MEC behavior and in suppressing the oncogenic activities of TGF-β. Interestingly, we find that inactivating noncanonical TGF-β effectors is sufficient in abrogating the ability of TGF-β to promote breast cancer progression in a manner somewhat reminiscent of that mediated by enforced c-Abl activation. These parallels raise a number of interesting questions, including (1) does c-Abl suppress mammary tumorigenesis by inhibiting EMT, or by stimulating MET; (2) does enforced c-Abl activation alter the selection or expansion of breast cancer stem cells; (3) does cytoplasmic or nuclear c-Abl activation mediate its antitumor activities; (4) does the initiation of oncogenic TGF-β signaling uncouple c-Abl from activation by TGF-β; and (5) do the tumor-suppressing activities of c-Abl require signaling inputs by TGF-β? These questions and their potential for directing the future development and implementation of c-Abl chemotherapies during mammary tumorigenesis are discussed below.

Although c-Abl activation clearly promotes the acquisition of epithelial phenotypes in normal and malignant MECs (fig. 3) [Allington et al., 2009], it remains to be determined whether this event reflects the ability of c-Abl to inhibit EMT, or conversely, to stimulate MET. This question also bears important clinical relevance because inhibiting EMT or stimulating MET are both likely to be most effective in preventing the exit of metastatic cells from the primary tumor; however, the induction of MET may be contraindicated should this process be found to play an essential role in promoting the outgrowth of micrometastatic lesions. Our findings show that c-Abl deficiency or inactivation both elicit EMT, while c-Abl activation induces a 'hyperepithelial' morphology that normalizes and reverts the phenotypes of metastatic breast cancer cells (fig. 3, 4) [Allington et al., 2009]. Along these lines, epithelial cells naturally tend to drift and acquire mesenchymal characteristics in response to extended 2D culture durations, suggesting perhaps that the process of becoming 'mesenchymal' may reflect a more energetically favorable and stable state than that needed to become 'epithelial'. Alternatively, the nearly infinite stiffness of 2D culture systems may serve as an aberrant signal that drives epithelial cells to acquire mesenchymal-like properties as a means to compensate and survive in extremely rigid microenvironments [Butcher et al., 2009; Erler and Weaver, 2009]. In fact, we observed microenvironmental tension to be sufficient in overriding the cytostatic activities of TGF-β, an event that was circumvented by CST-Abl expression [Allington et al., 2009]. Thus, our findings are consistent with the notion that enforced c-Abl activation stimulates MET, as opposed to simply inhibiting EMT. Future studies need to thoroughly address this issue, as well as to explore the potential linkage between c-Abl and known inducers of MET, including Pax-2, Cdx2 and frizzled-7 [Hugo et al., 2007].

Recently, human and mouse MECs were observed to acquire stem cell-like properties when stimulated to un-

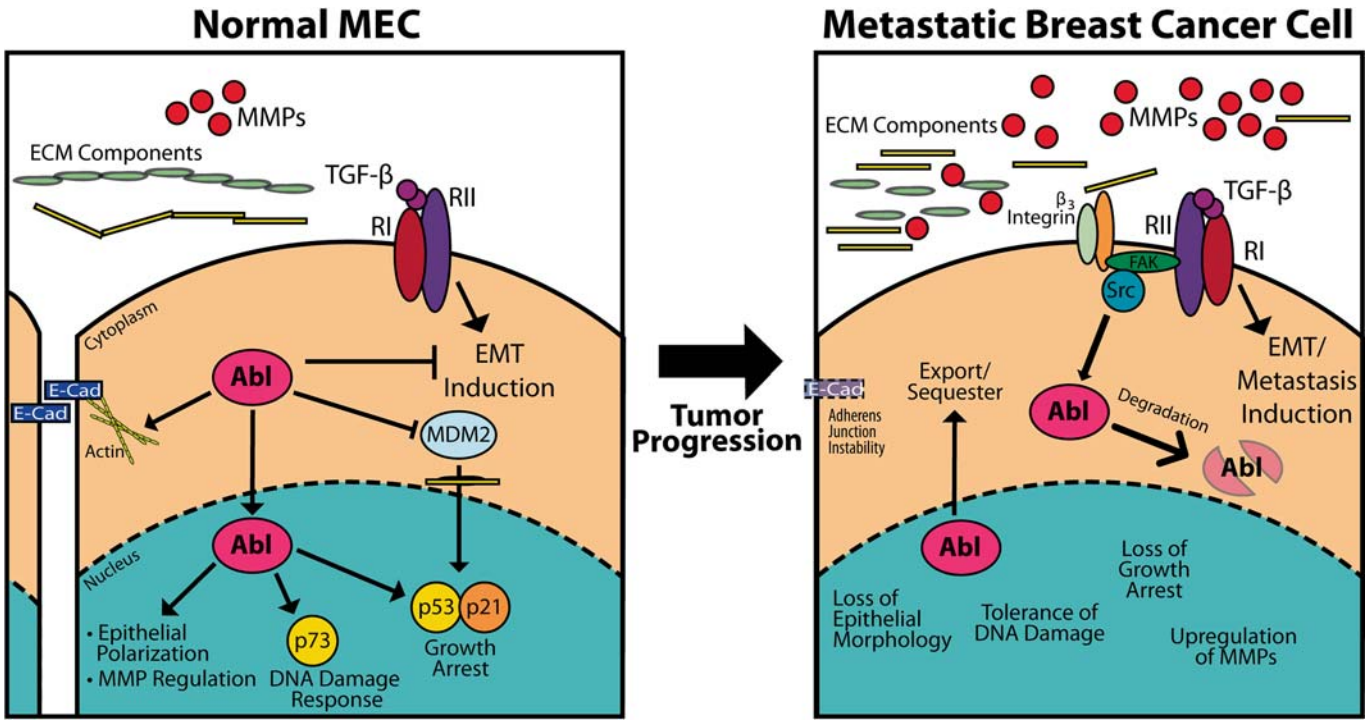


Fig. 5. Schematic depiction of the role of c-Abl in suppressing EMT and oncogenic TGF-β signaling in normal and malignant MECs. In normal MECs, c-Abl activation (1) maintains adherens junction stability and cortical actin architecture; (2) mediates growth arrest in response to TGF-β and DNA damage; and (3) represses MMP expression and secretion. As developing mammary neoplasms become more and more malignant, oncogenic TGF-β signaling in conjunction with focal adhesion complex activation (e.g., β_3-integrin/focal adhesion kinase/Src) may promote the degradation of c-Abl and its uncoupling from the tumor-suppressing activities of TGF-β. The loss of c-Abl function ushers in the initiation of EMT and its dissolution of adherens junctions, its production and activation of matrix metalloproteinases, and its circumvention of cytostasis induced by TGF-β and DNA damage, which collectively enhance breast cancer progression and metastatic dissemination to distant locales. RI/RII = TGF-β type I receptor/TGF-β type II receptor; E-cad = E-cadherin; MDM2 = murine double minute 2.

dergo EMT by TGF-β [Mani et al., 2008]. Mechanistically, upregulated Id1 expression may function in dictating whether TGF-β expands or contracts the pool of cancer stem cells [Tang et al., 2007], and consequently, whether TGF-β suppresses or promotes mammary tumorigenesis. Indeed, inhibiting TGF-β signaling pharmacologically in cancer stem cells elicited MET and their acquisition of less aggressive, more epithelial-like morphologies [Shipitsin et al., 2007]. Our demonstration that activated c-Abl phenotypically and morphologically reverts the malignant behaviors of late-stage breast cancer cells (fig. 3, 4) [Allington et al., 2009] raises 2 interesting questions – does enforced c-Abl activation suppress the selection and expansion of cancer stem cells, and conversely, does imatinib administration promote solid tumor progression by enlarging the population of stem cell-like progenitors in post-EMT carcinoma cells? Thus, future studies clearly need to investigate these important issues as well.

As mentioned above, the strong epithelial morphologies and phenotypes induced by c-Abl activation suggest that this PTK stimulates MET, presumably by functioning in the cytoplasm to affect adherens junctions and cytoskeletal architectures. However, in response to diverse extracellular stimuli (integrins), c-Abl is translocated to the nucleus where it functions in regulating DNA damage and mismatch repair responses [Lewis et al., 1996; Baskaran et al., 1997; Taagepera et al., 1998]. Moreover, c-Abl activation during DNA damage-induced apoptosis requires its coupling to the p53 relative, p73 [Yuan et al., 1999; Shaul, 2000]. Thus, the relative contributions of cytoplasmic and nuclear c-Abl in suppressing mammary tumorigenesis remains an important and unanswered question. With this idea in mind,

we observed the expression of CST-Abl to greatly enhance the induction of p21 expression by TGF-β [Allington et al., 2009] and to induce 4T1 organoids to express robust quantities of p73, suggesting that the morphological reversion of 4T1 acinar structures is partially dependent upon nuclear c-Abl signaling inputs [Allington and Schiemann, unpubl. observation]. Accordingly, we have found that CST-Abl expression is sufficient in resensitizing 4T1 cells to death induced by the DNA-damaging agent, 6-thioguanine (Allington and Schiemann, data not shown). Thus, while 6-thioguanine has previously failed as a single-agent chemotherapeutic for breast cancer, our findings suggest that combining enforced c-Abl activation with 6-thioguanine or other DNA damage-inducing agents may offer new inroads to alleviating breast cancer progression.

Finally, we found that TGF-β administration leads to a very transient activation of c-Abl in MECs [Allington et al., 2009], which is rapidly followed by their degradation of c-Abl in a manner that coincides with initiation of EMT [Allington and Schiemann, unpubl. observation]. Based on these findings, we speculate that the end result of c-Abl activation by TGF-β results in the Src-dependent degradation of c-Abl [Allington and Schiemann, unpubl. observation; Echarri and Pendergast, 2001; Woodring et al., 2002; Zhu and Wang, 2004], and consequently, in the acquisition of EMT phenotypes stimulated by TGF-β. This model clearly contrasts with the ability of TGF-β to couple to c-Abl activation via a phosphoinositide 3-kinase- and p21-activated kinase-2-dependent pathway in fibroblasts [Daniels et al., 2004; Wang et al., 2005; Wilkes and Leof, 2006]. Despite these disparate activities for c-Abl in MECs and fibroblasts, it remains plausible that the ability of TGF-β to induce EMT requires the inactivation and degradation of c-Abl in epithelial cells (fig. 5). Along these lines, we have recently observed TGF-β stimulation of EMT to drastically reduce the expression and activity of c-Abl in both the cytoplasm and nucleus of normal MECs. Moreover, the kinetics of c-Abl degradation mirrored that for the acquisition of focal adhesion complex signaling by TGF-β, and more importantly, chemotherapeutic targeting of focal adhesion complexes was sufficient in protecting c-Abl from degradation induced by TGF-β [Allington and Schiemann, unpubl. observation]. Interestingly, augmented [Galliher and Schiemann, 2006, 2007; Galliher-Beckley and Schiemann, 2008; Neil et al., 2008; Neil and Schiemann, 2008; Neil et al., 2009; Tian and Schiemann, 2009a; Wendt and Schiemann, 2009; Wendt et al., 2009b] and attenuated [Bhowmick et al., 2004a, 2004b; Cheng et al., 2005; Yang et al., 2008] TGF-β signaling in mammary carcinoma cells has been associated with disease progression, which raises a second interesting question – can c-Abl activation morphologically and phenotypically revert the malignant behaviors of breast cancer cells that can no longer respond to TGF-β? Future studies need to address this important issue, as well as determine how EMT dictates the expression, activation and localization of c-Abl in normal and malignant MECs. Indeed, answering these important questions may provide novel information capable of one day (1) staging and stratifying the treatment of mammary carcinomas based on their c-Abl and TGF-β signatures; and (2) enhancing our understanding of the 'TGF-β paradox' in promoting metastatic disease in breast cancer patients.

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FIBROMODULIN SUPPRESSES NUCLEAR FACTOR-κB ACTIVITY BY INDUCING THE DELAYED DEGRADATION OF IKBA *VIA* A JNK-DEPENDENT PATHWAY COUPLED TO FIBROBLAST APOPTOSIS

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Running Head: Fibromodulin increases IκBα stability

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Fibulin-5 (FBLN5) belongs to the Fibulin family of secreted extracellular matrix proteins and our laboratory first established FBLN5 as a novel target for TGF-β in fibroblasts and endothelial cells. To better understand the pathophysiology of FBLN5, we carried out microarray analysis to identify fibroblast genes whose expression were regulated by FBLN5 and TGF-β. In doing so, we identified fibromodulin (Fmod) as a novel target gene of FBLN5, and validated the differential expression of Fmod and twelve other FBLN5-regulated genes by semi-quantitative real-time PCR. Fmod belongs to the small leucine-rich family of proteoglycans, which are important constituents of mammalian extracellular matrices. Interestingly, parental 3T3-L1 fibroblasts displayed high levels of nuclear factor-κB (NF-κB) activity, while those engineered to express Fmod-constitutively exhibited significantly reduced NF-κB activity, suggesting that Fmod functions to inhibit NF-κB signaling. By monitoring alterations in the activation of NF-κB and the degradation of its inhibitor, IκBα, we demonstrate for the first time that Fmod contributes to the constitutive degradation of IκBα protein in 3T3-L1 fibroblasts. Mechanistically, we observed Fmod to delay the degradation of IκBα by promoting the *(i)* activation of c-Jun N-terminal kinase; *(ii)* inhibition of calpain and casein kinase 2 activity; and *(iii)* induction of fibroblast apoptosis. Taken together, our study identified a novel function for Fmod in directing extracellular signaling, particularly the regulation of NF-κB activity and cell survival.

Fibromodulin (Fmod) is a keratan sulfate proteoglycan that is highly concentrated in cartilage, tendon, skin, cornea and sclera (1). Fmod belongs to the small leucine-rich repeat (LRR) family of extracellular matrix (ECM) proteoglycans and glycoproteins, whose members also include decorin, biglycan, and lumican (2,3). Fmod plays a central role in organizing the structure of type I and II collagen fibrils, and its expression is ubiquitous in a variety of connective tissues, such as cartilage, tendons, ligaments, and dermal tissues (4,5). Along these lines, transforming growth factor-β (TGF-β) isoforms interact physically Fmod to regulate the fibrotic responses of several tissues, including the kidney, lung, and skin (6-8). Moreover, Fmod also affects the differentiation of tendon stem/progenitor cells by modulating the signals transduced by bone morphogenic proteins, ultimately enhancing tendon repair and formation *in vivo* (9). Thus, Fmod expression figures prominently in regulating tissue homeostasis in part by impacting the activities of TGF-β superfamily members.

TGF-β itself is a multifunctional cytokine that regulates tissue morphogenesis and differentiation by effecting cell proliferation and survival, and by altering the production of ECM proteins within cell and tissue microenvironments (10,11). In addition, our laboratory first identified the ECM protein Fibulin-5 (FBLN5) as a novel target gene for TGF-β in fibroblasts (12) and endothelial cells (12,13). Moreover, we established FBLN5 as a multifunctional signaling molecule that *(i)* regulates the proliferation, motility, and invasion of normal and malignant cells both *in vitro* and *in vivo* (12-14); *(ii)* antagonizes endothelial cell activities coupled to angiogenesis both *in vitro* and *in vivo* (13,14); *(iii)* inhibits the growth of fibrosarcomas in mice (14); and *(iv)* induces epithelial-mesenchymal transition (EMT) in normal and malignant mammary epithelial cells *via* a MMP-dependent mechanism (15). In an attempt to further our understanding of

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the pathophysiological functions of FBLN5, we characterized changes in the fibroblast transcriptome induced by constitutive FBLN5 expression. In doing so, we identified Fmod as a novel target gene of FBLN5 in fibroblast cells. Whereas previous studies of Fmod action have been performed primarily in connective tissues (16) and endothelial cells (17), we show here that Fmod expression in fibroblasts potently suppressed their activation of nuclear factor-κB (NF-κB) by preventing the degradation of IκBα *via* a c-Jun N-terminal kinase (JNK)-, casein kinase-2 (CK2)-, and calpain-dependent pathway that serves in diminishing fibroblast survival.

EXPERIMENTAL PROCEDURES

Cell lines and Reagents – Normal murine 3T3-L1 fibroblasts were obtained from ATCC (Manassas, VA) and cultured as described previously (12). 3T3-L1 fibroblasts were engineered to stably express either GFP or Fmod using a bicistronic retroviral vector (pMSCV-IRES-GFP) as described previously (12). The NF-κB promoter-driven luciferase reporter was provided by Dr. John M. Routes (Medical College of Wisconsin, Milwaukee, WI). The generation of 3T3-L1 fibroblasts that lacked Fmod expression was accomplished by their transduction with lentiviral particles that encoded for either a scrambled (*i.e.,* non-silencing) or Fmod-directed shRNA in pLKO.1; Thermo Scientific, Huntsville, AL). The generation of pLKO.1 lentiviral particles and their transduction into 3T3-L1 fibroblasts was accomplished as described previously (18,19), while the extent of Fmod-depletion was monitored by immunoblotting whole-cell extracts prepared from parental (*i.e.,* non-silencing shRNA) and Fmod-deficient 3T3-L1 fibroblasts with anti-Fmod antibodies.

Microarray Analysis and Semi-Quantitative Real-Time PCR Assays – Total RNA from parental (*i.e.,* GFP) and FBLN5-expressing 3T3-L1 fibroblast cells was purified using the RNeasy Plus Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's recommendations. The creation of polyclonal clonal populations of 3T3-L1 cells that constitutively expressed FBLN5 and its role in promoting fibroblast proliferation and MAP kinase activation were described previously (12). In some experiments, GFP- or FBLN5-expressing 3T3-L1 fibroblasts were stimulated with TGF-β1 (5 ng/ml). RNA samples were reverse-transcribed and labeled for microarray analysis using standard techniques (20,21). For real-time PCR, cDNAs were synthesized by iScript reverse-transcription (Bio-Rad, Hercules, CA), which then were diluted 10-fold in H_2O and employed in semi-quantitative real-time PCR reactions (25 μl) using the SYBR Green system (Bio-Rad) supplemented with 5 μl of diluted cDNA and 0.1 μM of oligonucleotide pairs provided in Supplemental Table S1. PCR reactions were performed and analyzed on a Bio-Rad Mini-Opticon detection system, and differences in RNA concentrations were monitored by normalizing individual gene signals to their corresponding GAPDH signals.

Immunoblot Assays – Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were lysed and solubilized in Buffer H/Triton X-100 (22) for 30 min on ice. Clarified whole-cell extracts were resolved on 10% SDS-PAGE gels, transferred electrophoretically onto nitrocellulose membranes, and blocked in 5% milk before incubation with the following primary antibodies (dilutions): (*a*) anti-Fmod (1:1000; Santa Cruz Biotechnology, Santa Cruz, CA); (*b*) anti-IκBα (1:3000; Santa Cruz Biotechnology); (*c*) anti-phospho-IκBα (1:1000; Cell Signaling, Danvers, MA); (*d*) anti-phospho-ERK (1:500; Cell Signaling); (*e*) anti-phospho-p38 (1:500; Cell Signaling); (*f*) anti-phospho-JNK (1:500; Cell Signaling); (g) anti-p65 (1:1,000; Santa Cruz Biotechnology); and (h) anti-histone H1 (1:200; Santa Cruz Biotechnology). The resulting immunocomplexes were visualized by enhanced chemiluminescence. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies (1:1000; Rockland Immunochemicals, Gilbertsville, PA). Where specified, parental (*i.e.,* GFP), Fmod-expressing, or Fmod-deficient (Fmod shRNA) 3T3-L1 fibroblasts were incubated for various times in the absence or presence of the *(i)* protein synthesis inhibitor, cycloheximide (CHX); *(ii)* proteosome inhibitor, MG-132; *(iii)* JNK inhibitor, SP600125; *(iv)* calpain inhibitor, EST (Calbiochem, San Diego, CA); and *(v)* CK2 inhibitor, TBBz (Calbiochem) at the provided concentrations.

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Luciferase Reporter Gene Assays – Analysis of luciferase activity driven by a synthetic NF-κB promoter was accomplished as described (23). Briefly, parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts (25-30,000 per well) were cultured overnight onto 24-well plates, and subsequently were transiently transfected the following morning by overnight exposure to LT1-liposomes (Mirus, Madison, WI) containing pNF-κB-luciferase cDNA (300 ng/well) and CMV-β-gal cDNA (50 ng/well,) which was used to control for differences in transfection efficiency. Afterward, resulting luciferase and β-gal activities contained in detergent-solubilized cell extracts were determined. As above and where specified, parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of the inhibitors to JNK (SP600125), calpain (EST), and CK2 (TBBz). Data are the mean (± SE) luciferase activities of at least 3 independent experiments normalized to untreated cells.

NF-κB Biotinylated Oligonucleotide Capture Assay - DNA binding activity of NF-κB was monitored in parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts as described previously (23). Briefly, the cells were incubated for 24 h in the absence or presence of JNK inhibitor (SP600125), and subsequently were collected and fractionated into cytoplasmic and nuclear extracts using a Nuclear Extraction Kit according to the manufacturer's instructions (Chemicon). NF-κB binding activity was determined by incubating 60 μg of nuclear extract with 1 μg of biotinylated double-stranded DNA oligonucleotides that contained a NF-κB consensus sequence site under continuous rotation at 4˚C (forward probe, 5′-GATTAGGGACTTTCCGCTGGGGACTTTCCA GTCGA; reverse probe, 5′-TCGACTGGAAA GTCCCCAGCGGAAAGTCCCTAGATC). The resulting NF-κB:oligonucleotide complexes were captured by addition of streptavidin-agarose beads (Pierce) and collected by microcentrifugation. Washed complexes were fractionated through 10% SDS-PAGE before their immobilization to nitrocellulose membranes, which were subsequently probed with anti-p65 antibodies (1:500). Differences in extract loading were monitored by immunoblotting 25 μg of resolved nuclear extract aliquots with antibodies against histone H1 (1:200).

Cell Biological Assays – The effect of Fmod on calpain activity in 3T3-L1 fibroblasts was determined using the Calpain-Glo™ Protease Assay kit (Promega, Madison, WI) according to the manufacturer's recommendations. Briefly, 3T3-L1 fibroblasts (30,000 cells/well in 96-well plates) were lysed and mixed with proluminescent calpain substrate, and the resulting luminescence was measured as previously described (12). Along these lines, the effect of Fmod on 3T3-L1 fibroblast apoptosis was determined using two complementary methods. First, 3T3-L1 fibroblasts (10,000 cells/well) were incubated for 24 h in the absence or presence of JNK inhibitor SP600125 prior to addition of the proluminescent caspase-3/7 DEVD-aminoluciferin substrate as instructed by the Caspase-Glo® Assay kit (Promega; (24)). Second, parental (*i.e.,* GFP), Fmod-expressing, or Fmod-deficient (*i.e.,* Fmod shRNA) 3T3-L1 cells were again subjected to JNK inhibitor prior to their processing for TUNEL Assays according to the manufacturer's recommendations (Invitrogen, Carlsbad, CA).

RESULTS

Fmod is a Novel Target Gene of FBLN5 in 3T3-L1 Fibroblasts – Our laboratory first established FBLN5 as a novel gene target for TGF-β in fibroblasts and endothelial cells (12). Moreover, we demonstrated that constitutive FBLN5 expression was sufficient to enhance 3T3-L1 cell proliferation and activation of MAP kinases (12). In an effort to enhance our understanding of the pathophysiological actions of FBLN-5, we characterized changes in the fibroblast transcriptome induced by constitutive FBLN5 expression, or by constitutive FBLN5 expression plus added TGF-β1. Afterward, total RNA was collected and prepared for hybridization to Affymetrix microarrays, which enabled us to identify ~1200 genes whose expression was controlled by constitutive FBLN-5 expression. The altered expression of 13 genes was confirmed by semi-quantitative real-time PCR (Table 1), including that of Fmod (NCBI accession # NM_021355). Here we limited our analyses to Fmod since this ECM protein and

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FBLN5 both play prominent roles in regulating cell-cell and cell-matrix signaling in ECM. Interestingly, FBLN5 only coupled to Fmod expression in 3T3-L1 fibroblasts, but not in normal (NMuMG) or malignant (MDA-MB-231) mammary epithelial cells, or in endothelial (MB114) cells (see supplemental Fig. S1). Collectively, these findings identify Fmod as a novel and fibroblast-specific gene target of FBLN5.

Fmod Suppresses NF-κB Activity by Stabilizing IκBα – In order to study the function of Fmod, we first established Fmod-expressing fibroblasts using a retroviral expression system according to our published procedures (12). As expected, fibroblasts transduced with Fmod-encoding viral particles constitutively-expressed Fmod proteins at significantly higher levels as compared to their parental (*i.e.,* GFP) counterparts (Fig. 1*A*). We then measured the production of luciferase whose expression was driven by Smad2/3 or AP1 transcription factors in these cells before and after their stimulation of with TGF-β1 because this cytokine is known to readily interact with Fmod (25). In doing so, we observed Fmod-expressing fibroblasts to exhibit significantly elevated luciferase expression driven by the Smad2/3 and AP1 transcription factors both basally and in response to TGF-β (*data not shown*). We also examined the effects of Fmod in altering the expression of luciferase driven by NF-κB. In comparison to parental (*i.e.,* GFP) fibroblasts, those engineered to express Fmod exhibited a 75% reduction of luciferase activity directed by NF-κB (Fig. 1*B*). Along these lines, the ability of NF-κB to mediate induce the expression of matrix metalloproteinases (MMP)-2 and -9 in fibroblasts has previously been established (26), and as such, Fmod-mediated inhibition of NF-κB activity would be anticipated to reduce fibroblast expression of MMPs 2 and 9. Accordingly, Fmod-expressing fibroblasts did indeed express significantly reduced levels of MMPs 2 and 9 as compared to their parental (*i.e.,* GFP) counterparts (see supplemental Fig. S2).

NF-κB activity is regulated primarily by its interaction with inhibitory κB (IκB) proteins (27). Extracellular signals that stimulate NF-κB must first induce the release IκB proteins from NF-κB, thereby allowing for its nuclear translocation and regulation of gene expression. This initial dissociation event requires the activation of IκB kinases (IKKs), which phosphorylate IκB proteins and lead to their subsequent release from NF-κB, ubiquitination, and proteosomal degradation (27). In light of the dramatic reduction in NF-κB measured in Fmod-expressing fibroblasts, we immunoblotted whole-cell extracts prepared from parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts with anti-IκBα antibodies. Fig. 1*C* shows that Fmod-expressing fibroblasts contain significantly higher quantities of IκBα as compared to their parental counterparts, a finding consistent with the diminished NF-κB activity measured in Fmod-expressing fibroblasts. Interestingly, although IκBα levels were considerably higher in Fmod-expressing *versus* parental fibroblasts, we observed the phosphorylation status of IκBα to be equivalent between these cell lines (Fig. 1*C*). This finding suggests that the ability of Fmod to inhibit NF-κB activity may reflect dysregulated IκBα dynamics. As such, we monitored the stability of IκBα proteins in parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts before and after their treatment with cycloheximide (CHX), which inhibits proteins synthesis and enables assessment of the rate of IκBα turnover. As shown in Fig. 2*A*, IκBα was rapidly degraded in parental (*i.e.,* GFP) 3T3-L1 fibroblasts treated with CHX; however, this same experimental treatment elicited little-to-no change in the levels of IκBα detected in Fmod-expressing fibroblasts. Likewise, administration of MG-132, which inhibits proteosomal-mediated protein degradation, also failed to alter the levels IκBα in Fmod-expressing fibroblasts (Fig. 2*B*). Collectively, these findings suggest that Fmod inhibits NF-κB activity by preventing the degradation of IκBα. To more thoroughly investigate the merits of this supposition, we transduced Fmod-expressing 3T3-L1 fibroblasts with scrambled or Fmod-directed shRNAs to neutralize the activities of constitutive Fmod expression. The extent of Fmod-deficiency mediated by individual Fmod-directed shRNAs was monitored by immunoblotting with antibodies against Fmod (see supplemental Fig. S3 and Table S2). Based on these analyses, polyclonal populations of 3T3-L1 cells that stably expressed Fmod shRNA2 were selected and used for all subsequent Fmod-deficiency studies. As shown in Fig. 2*C*, elevating Fmod expression (*i.e.,* Fmod) prevented IκBα degradation in CHX-

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treated fibroblasts, a reaction that was readily reversed in fibroblasts depleted of Fmod expression (*i.e.,* shFmod). Moreover, the enhanced stability of IκBα was specific to Fmod expression because the introduction of nonsilencing shRNA (*i.e.,* scram) failed to restore IκBα degradation (Fig. 2*C*). Taken together, these findings indicate that Fmod repressed the activation of NF-κB by increasing the stability of IκBα.

Fmod Stabilizes IκBα by Activating JNK – Mammalian mitogen-activated protein (MAP) kinases play important roles in many different signaling pathways, including the control of gene expression, and of cell proliferation, differentiation, and apoptosis (28,29). NF-κB and JNK are two key regulators of the pathophysiology of cells, and crosstalk between these two signaling molecules has been reported in the scientific literature (30-32). These connections prompted us to test whether the ability of Fmod to inhibit NF-κB transpired through MAP kinases, particularly JNK. In doing so, we monitored the activation status of individual MAP kinases by immunoblotting extracts prepared from parental (*i.e.,* GFP) or Fmod-expressing fibroblasts with phospho-specific anti-MAP kinase antibodies. Fig. 3*A* shows that while Fmod expression failed to activate ERK1/2 and elicited only modest activation of p38 MAPK, this ECM protein did significantly stimulate the phosphorylation of JNK in a manner that correlated with elevated IκBα expression. Thus, JNK activation may underlie diminished NF-κB activity in Fmod-expressing fibroblasts. To address this hypothesis, we treated parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts with the JNK inhibitor, SP600125, prior to monitoring changes in IκBα expression. Interestingly, inhibiting JNK activity elicited only modest alterations in IκBα levels in parental (*i.e.,* GFP) fibroblasts; however, this same experimental treatment induced a dramatic reduction of IκBα in Fmod-expressing cells (Fig. 3*B*), suggesting that Fmod-mediated activation of JNK prevents IκBα degradation. Moreover, this finding contrasts sharply with the failure of inhibitors against p38 MAPK (SB203580) or MEK1/2 (U0126) to alter IκBα expression in Fmod-expressing 3T3-L1 fibroblasts (*data not shown*). Along these lines, we observed SP600125 to dose-dependently increase NF-κB activity specifically in Fmod-expressing fibroblasts, but not in their parental (*i.e.,* GFP) counterparts (Fig. 3*C*). Similarly, transient expression of Fmod in 3T3-L1 fibroblasts significantly reduced their resting levels of NF-κB activity by ~50%, an effect that was significantly reversed following administration of SP600125 (see supplemental Fig. S4). We also observed significantly less NF-κB to be captured from nuclear fractions of Fmod-expressing 3T3-L1 cells as compared to their GFP-expressing counterparts (Fig. 3*D*). Moreover, inhibiting JNK activity augmented the activation and nuclear accumulation of NF-κB specifically in Fmod-expressing 3T3-L1 cells (Fig. 3*D*). To further address the role of JNK in prolonging half-life of IκBα, we also expressed a constitutively-active mutant of MEKK1 (CAMEKK1), which couples to JNK activation (33). As expected, elevated JNK phosphorylation was readily detected in fibroblasts transiently transfected with CAMEKK1 as compared to mock (*i.e.,* empty pcDNA) transfected cells (Fig. 3*E*). Moreover, expression of CAMEKK1 in 3T3-L1 fibroblasts reduced their NF-κB activity by ~75%, a response that was partially neutralized by administration of SP600125 (Fig. 3*F*). Thus, enforced activation of JNK by expression of CAMEKK1 in parental (*i.e.,* GFP) fibroblasts recapitulated the actions of Fmod on NF-κB signaling in fibroblasts. Finally, to determine the role of Fmod in promoting JNK-mediated IκBα stabilization, we once again depleted constitutive Fmod expression in 3T3-L1 fibroblasts using shRNAs directed against this ECM protein. As shown in Fig. 3*G*, administering SP600125 to Fmod-expressing cells (*i.e.,* Fmod and Fmod/scram) readily promoted IκBα degradation; however, this same treatment protocol failed to effect IκBα levels in cells depleted of Fmod. Taken together, these findings strongly suggest that Fmod expression stimulates JNK and its ability to prolong the half-life of IκBα, leading to reduced NF-κB activity in fibroblasts.

Constitutive Degradation of IκBα in Fibroblasts is Mediated by Calpain – Several reports established calpain as a mediator of constitutive IκBα degradation (34-36). In light of our findings that demonstrated the ability of Fmod to suppress IκBα degradation, we next determined whether calpain activity was differentially regulated be-

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tween parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts using the Calpain-Glo™ Protease Assay. As shown in Fig. 4*A*, Fmod-expressing 3T3-L1 fibroblasts exhibited 40% less calpain activity as compared to their parental (*i.e.,* GFP) counterparts, a finding consistent with the ability of Fmod to enhance IκBα stability (Fig. 1-3). As such, we determined whether administering the calpain inhibitor, EST, could alter NF-κB activity in a JNK-dependent manner. Fig. 4*B,C* shows that inhibiting calpain alone or in combination with JNK had no effect on NF-κB activity in parental (*i.e.,* GFP) fibroblasts. In stark contrast, NF-κB activity was readily induced in Fmod-expressing cells treated with the JNK inhibitor, SP600125. More importantly, the effects of JNK inactivation on NF-κB activity were readily reversed by administration of the calpain inhibitor, EST (Fig. 4, *B* and *C*). Identical findings were obtained by treating Fmod-expressing fibroblasts with a second independent calpain inhibitor, calpain inhibitor III (see supplemental Fig. S5). Taken together, these findings suggest that elevated expression of Fmod inhibited calpain protease activity and its degradation of IκBα, which manifests significantly reduced NF-κB activity in 3T3-L1 fibroblasts.

Along these lines, the ability of calpain to degrade IκBα has been associated with the activation of CK2 (36,37). Our findings thus far suggest that parental 3T3-L1 fibroblasts house likely high levels of active CK2, which couples to calpain activation and the rapid degradation of IκBα, resulting in constitutive NF-κB activity. In contrast, our findings also predict that Fmod-expressing 3T3-L1 fibroblasts likely house negligible levels of active CK2 that cannot adequately couple to calpain activation, and as such to elevated NF-κB activity. Thus, constitutively-active NF-κB activity in parental (*i.e.,* GFP) fibroblasts is predicted to be inhibited by administration of CK2 antagonists, while their Fmod-expressing counterparts are predicted to be refractory to CK2 inhibition. To determine the validity of these predictions, we measured NF-κB luciferase activity in parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts before and after their treatment with the CK2 inhibitor, TBBz. Fig. 4*D* shows that NF-κB activity in Fmod-expressing fibroblasts was insensitive to administration of TBBz; however, this same experimental regimen significantly inhibited the transcriptional activity of NF-κB in parental (*i.e.,* GFP) fibroblasts. Moreover, identical findings were obtained by treating parental (*i.e.,* GFP) and Fmod-expressing fibroblasts with a second independent CK2 inhibitor, TBCA (see supplemental Fig. S6). Collectively, these findings suggest that the constitutive turnover of IκBα is mediated by a CK2:calpain signaling axis that is inactivated in Fmod-expressing fibroblasts in a JNK-dependent manner.

Fmod Induces Fibroblast Apoptosis – Recent findings have linked CK2 to the regulation of cell survival in part *via* its ability to promote IκBα degradation and NF-κB activation (38). Because constitutive Fmod expression promotes JNK activation coupled to reduced calpain activity and enhanced IκBα stability, we investigated the effect of Fmod on fibroblast survival using the Caspase-Glo™ 3/7 Assay kit which showed that Fmod-expressing fibroblasts possessed significantly higher caspase-3/7 activities as compared to parental (*i.e.,* GFP) fibroblasts (Fig. 5*A*). In contrast to parental (*i.e.,* GFP) fibroblasts, SP600125 administration significantly reduced caspase-3/7 activity in Fmod-expressing fibroblasts, suggesting that Fmod-mediated activation of JNK couples to caspase-3/7 activation. We also determined the relative reliance of caspase-3/7 activation on Fmod by once again depleting its expression in Fmod-expressing cells *via* introduction of Fmod-directed shRNA. Fig. 5*B* clearly shows that Fmod expression was essential for JNK-dependent caspase-3/7 activation in 3T3-L1 fibroblasts. Along these lines, we observed Fmod-expressing 3T3-L1 cells to possess elevated TUNEL staining as compared to their parental and Fmod-depleted counterparts (Fig. 5*C*). As above, inhibiting JNK activity significantly reduced TUNEL staining only in Fmod-expressing 3T3-L1 cells (Fig. 5*C*), suggesting that Fmod-mediated activation of JNK promotes apoptosis in fibroblasts. Collectively, these findings demonstrate that Fmod expression is necessary for the activation of JNK (Figure 3) and, consequently, for activation of caspase 3/7 to initiate fibroblast apoptosis (Figure 5).

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DISCUSSION

NF-κB is a ubiquitously expressed transcription factor that governs the transcription of genes involved in immune responses, angiogenesis, cell transformation, invasion, migration, apoptosis, and cell cycle (39). As such, the inappropriate activation of NF-κB has been linked to variety of inflammatory and autoimmune diseases, and of human malignancies (40). The signaling of NF-κB is normally suppressed by a family of inhibitory molecules termed IκB proteins, such as IκBα that bind NF-κB and prevents its activation in the cytoplasm. In response to a variety of stimuli, including proinflammatory cytokines (*e.g.,* TNF-α or IL-1), T and B cell mitogens, lipopolysaccharide, viral infections, and cellular stresses (*e.g.,* ionizing radiation or chemotherapies) (41,42), IκBα molecules are rapidly degraded to facilitate the activation and nuclear translocation of NF-κB (39). The extent to which ECM molecules regulate NF-κB activity and the molecular mechanisms whereby these events transpire remain incompletely understood. To this end, we show here for the first time that Fmod is a novel gene target of FBLN5 (Table 1), which itself functions as an effector of TGF-β. More importantly, we established a novel Fmod signaling axis that significantly enhances the stability and accumulation of IκBα proteins *via* the activation of JNK, which suppresses the ability of CK2 to stimulate calpain and its induction of IκBα turnover. The net effect of these Fmod-dependent events manifests as reduced NF-κB activity and elevated fibroblast apoptosis. This response differs significantly from canonical NF-κB activation, which transpires through IKK-dependent phosphorylation of IκBα and its subsequent ubiquitination and proteosomal degradation (35,41-44). In fact, our Fmod findings are more aligned with the regulatory events employed in resting cells to govern the balance between IκBα expression and NF-κB activity (43,45). Indeed, IκBα is rapidly degraded to elicit constitutive NF-κB activity in a variety of cells, including in B and T cells (34,46), in hepatocytes (47), and as shown here, in fibroblasts. Moreover, our findings established a novel signaling axis that enables Fmod to suppress NF-κB activity *via* a JNK-dependent mechanism operant in stabilizing IκBα expression. Moreover, we demonstrated the ability of this Fmod signaling axis to negate the pro-survival signals generated by CK2 and its stimulation NF-κB *via* calpain-mediated degradation of IκBα. Future studies need to address how altered fibroblast survival impacts stromal homeostasis and its paracrine activity on neighboring epithelial cells.

Along these lines, lysyl oxidases (LOX) comprise a five member gene family of copper-dependent amine oxidases that function in crosslinking collagens to elastin in the ECM, thereby increasing the tensile strength and structural integrity of tissues during embryonic development and organogenesis, and during the maintenance of normal tissue homeostasis (48,49). Interestingly, recent findings found that LOX expression in Ras-transformed NIH3T3 cells inhibited the activity of NF-κB in part by stabilizing IκBα (50). In addition, FBLN5 interacts physically with the LOX family members LOXL1, LOXL2, and LOXL4 (51,52), which points to a common link between FBLN5, Fmod, and LOXs in contributing to the suppression of NF-κB activity by increasing the half-life of IκBα. Our findings also suggest that these ECM proteins fulfill both structural and signaling functions within the stromal compartment. The precise interplay between these ECM components in regulating the pathophysiological actions of NF-κB await further experimentation.

At present, there exist two major pathways coupled to the turnover of IκBα, namely proteosome- and calpain-dependent degradation of IκBα. Overexpression of the epidermal growth factor receptor family member Her-2/neu induces constitutive NF-κB activity through a PI3-kinase/Akt-dependent pathway that promotes calpain-mediated degradation of IκBα (53). Moreover, it has been reported that IκBα degradation in B cells transpires exclusively *via* a calcium/calpain-dependant pathway, but not *via* the ubiquitin/proteosome-dependent pathway (35). And finally, CK2-dependent phosphorylation of the PEST domain in IκBα facilitates its destruction by calpain (36). Our study reinforces the importance of calpain in regulating constitutive NF-κB activity in fibroblasts, as well as identifies a novel Fmod:JNK-mediated signaling axis operant in neutralizing the pro-survival functions of CK2 and

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calpain. Our findings, together with those mentioned above, suggest that these players may represent a general mechanism to fine tune NF-κB signaling in resting cells and tissues, particularly their ability to adapt to altered survival signals. Upregulated expression of Fmod and other proteoglycans has been detected during the repair and remodeling of wounded collagen matrices, a response that transpires in the absence of obvious inflammatory reactions (54). Moreover, we previously associated FBLN5 to enhanced wound healing *in vivo* (55), and as such, future studies need to investigate the relative contribution of Fmod to these events, and to the initiation and resolution of tissue inflammation and fibrotic responses.

As mentioned previously, Fmod belongs to the LLR family of ECM proteoglycans and glycoproteins typically found in connective tissues, including cartilage, tendon, skin, cornea and sclera (1). Fmod plays a significant role in collagen assembly and maintenance, and Fmod-deficient mice display abnormally thin type I collagen fibrils in their tendons, which increases their occurrence of arthritis (56). Besides its structural functions, Fmod also transduces signals by interacting with a variety of ECM and secreted molecules. Indeed, recent work demonstrated the ability of Fmod to stimulate the complement cascade through its physical interaction with C1q (57). Interestingly, while other ECM molecules such as laminin and decorin also bind C1q, only Fmod is capable of inducing complement activation and sustained inflammatory reactions. Thus, studies aimed at determining the interplay between Fmod and NF-kB in directing inflammation also appear warranted. Likewise, the regulatory roles of NF-κB in cancer biology, including its coupling to tissue invasion, migration, and metastasis, have been thoroughly established (58,59). Interestingly, the expression of Fmod transcripts are significantly reduced in metastatic tumors as compared with their nonmetastatic counterparts, particularly tumors originating in the breast (60,61) and prostate (62-64). In addition, Oncomine analyses of Fmod expression showed the expression of this proteoglycan to be reduced at metastatic sites relative to nonmetastatic lesions in gastric cancers, head and neck cancers, and sarcomas *(data not shown)*. These analyses also found aberrantly low Fmod expression to associate with reduced overall survival rates in patients with cancers of the brain, breast, lung, and blood *(data not shown)*. Thus, future studies need to investigate the specific contributions of diminished Fmod expression that elicit the acquisition of metastatic phenotypes in human tumors.

Finally, Fmod clearly functions in regulating the dynamics between developing carcinoma and their accompanying stroma (65). In particular, desmoplastic reactions in carcinomas elicit elevated interstitial fluid pressure that diminishes efficient nutrient and gaseous transfer, as well as reduces the delivery of cancer chemotherapeutics (66). The inflammatory environment of developing carcinomas is thought to induce stromal production of Fmod, leading to the acquisition of dense and rigid tissue architectures (65). Future clearly studies need to address the chemotherapeutic potential of augmenting or attenuating Fmod action in developing carcinomas as a novel means to improve the efficacy of cancer chemotherapeutics.

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FOOTNOTES

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The abbreviations used are: CHX, cycloheximide; CK2, casein kinase 2; ECM, extracellular matrix; FBLN5, fibulin-5; Fmod, fibromodulin; JNK, c-Jun N-terminal kinase; LOX, lysyl oxidase; LRR, leucine-rich repeat; MAP, mitogen-activated protein; NF-κB, nuclear factor-κB; TGF-β, transforming growth factor-β.

FIGURE LEGENDS

FIGURE 1. **Fmod expression suppresses NF-κB activity in fibroblasts.** *A,* Immunoblot analysis of parental (*i.e.,* GFP) or Fmod-expressing 3T3-L1 cell extracts with anti-Fmod antibodies showed that Fmod protein expression was abundant in fibroblasts transduced by bicistronic retroviral vector (pMSCV-IRES-GFP) encoding for murine Fmod. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Data are representative images from a single experiment that was performed 4 times. *B,* Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal. Forty-eight hours later, luciferase activities were measured and normalized to that of β-gal. Data are the mean (± SE) ratios of luciferase:β-gal activity observed in 3 independent experiments completed in triplicate (⋆, $P < 0.05$). *C,* Whole-cell extracts prepared from parental (*i.e.,* GFP) and Fmod-expressing fibroblasts were fractionated through 10% SDS-PAGE and immobilized onto nitrocellulose. The expression level of IκBα, phospho-IκBα, and β-actin was monitored by immunoblot analysis. Data are representative images from a single experiment that was performed 4 times.

FIGURE 2. **Fmod prolongs the half-life of IκBα in fibroblasts.** Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for varying times (0-24 h) in the absence or presence of cycloheximide (CHX, 50 μg/ml; *panel A*), or MG-132 (20 μM; *panel B*) as indicated. Afterward, whole-cell extracts were prepared, fractionated, and immobilized to nitrocellulose prior to their immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are representative images from a single experiment that was performed 4 times. *C,* Parental (*i.e.,* GFP), Fmod-expressing (Fmod, scram), or Fmod-depleted (shFmod) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of cycloheximide (CHX, 50 μg/ml). The expression levels of IκBα was visualized by immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed 4 times with similar results.

FIGURE 3. **Fmod stabilizes IκBα by activating JNK.** *A,* Whole-cell extracts prepared from parental (*i.e.,* GFP) and Fmod-expressing cells were fractionated through 10% SDS-PAGE and immobilized onto nitrocellulose. Immunoblot analysis with antibodies against IκBα, phospho-ERK (p-ERK), phospho-JNK (p-JNK), phospho-p38 MAPK (p-p38) showed that Fmod specifically couples to JNK activation. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed 3 times with identical results. *B,* Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated in the absence or presence of the JNK inhibitor, SP600125 (10 μM) for 24 h. Afterward, the expression levels of IκBα were detected by immunoblot analysis using anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. Data are from a representative experiment that was performed 3 times with similar results. *C,* Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with the JNK inhibitor (SP600125) as indicated. Data are the mean (± SE) ratios of

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luciferase:β-gal activity observed in 3 independent experiments completed in triplicate (★, $P < 0.05$). *D,* Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h with the JNK inhibitor (SP600125) as indicated, and nuclear extracts were prepared and incubated with biotinylated NF-κB oligonucleotide probes. Afterward, p65/RelA:oligonucleotide complexes were captured with streptavidin-agarose beads and visualized by immunoblotting with antibodies against total p65/Rel A. Data are from a representative experiment that was performed three times with similar results. *E,* Control (*i.e.,* pcDNA) or constitutively-active MEKK1 (CAMEKK1) were transiently transfected into 3T3-L1 cells, whose subsequent level of JNK phosphorylation was monitored by immunoblot analysis using anti-phospho-JNK antibodies (p-JNK). Differences in protein loading were controlled by immunoblotting for β-actin. Images are from a single experiment that was performed 4 times with identical results. *F,* Control (*i.e.,* pcDNA) and constitutively-active MEKK1 (CAMEKK1) were co-transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with the JNK inhibitor, SP600125 (25 μM) as indicated. Data are the mean (± SE) ratios of luciferase:β-gal activity observed in 3 independent experiments completed in triplicate (★, $P < 0.05$). *G,* Fmod-expressing (Fmod, scram) and Fmod-depleted (shFmod, GFP) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of JNK inhibitor, SP600125 (25 μM). The expression levels of IκBα were analyzed by immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with anti-β-actin antibodies. Data are from a representative experiment that was performed 3 times with similar results.

FIGURE 4. **Constitutive degradation of IκBα in fibroblasts is mediated by calpain.** *A,* Calpain activity was measured using the Calpain-Glo™ Protease Assay kit according to the manufacturer's protocol (Promega). Data are the mean (± SE; n=3) calpain activity relative to that measured in parental (*i.e.,* GFP) cells. *B,* Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with inhibitors to JNK (SP600125, 25 μM), Calpain (EST, 50 μM), or both compounds as indicated. Data are the mean (± SE) ratios of luciferase:β-gal activity observed in 3 independent experiments completed in triplicate (★, $P < 0.05$). *C,* Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated for 24 h with inhibitors to JNK (SP600125, 25 μM), Calpain (EST, 50 μM), or both compounds as indicated. Afterward, whole-cell extracts were prepared, fractionated, and immobilized to nitrocellulose prior to their immunoblotting with anti-IκBα antibodies. Differences in protein loading were monitored by reprobing stripped membranes with antibodies against β-actin. *D*, Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were transiently transfected with pNF-κB-luciferase and pCMV-β-gal, and subsequently were incubated for 24 h with the CK2 inhibitor, TBBz as indicated. Data are the mean (± SE) ratios of luciferase:β-gal activity observed in 3 independent experiments completed in triplicate (★, $P < 0.05$).

FIGURE 5. **Fmod induces fibroblast apoptosis.** *A,* Parental (*i.e.,* GFP) and Fmod-expressing 3T3-L1 fibroblasts were incubated in the absence or presence of JNK inhibitor, SP600125 (25 μM) for 24 h, at which point Caspase 3/7 activity was measured using Caspase-Glo™ 3/7 Assay according to the manufacturer's recommendations (Promega). Data are the mean (± SE) of activity observed in 3 independent experiments completed in triplicate (★, $P < 0.05$). *B,* Fmod-expressing (scram) and Fmod-depleted (shFmod) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of JNK inhibitor, SP600125 (25 μM). Afterward, caspase 3/7 activity was measured as above. Data are the mean (± SE) activity observed in 3 independent experiments completed in triplicate (★, $P < 0.05$). *C*, Fmod-expressing (Fmod, scram) and Fmod-depleted (GFP, shFmod) 3T3-L1 fibroblasts were incubated for 24 h in the absence or presence of JNK inhibitor, SP600125 (25 μM). Afterward, TUNEL staining was performed. Data are from a representative experiment that was performed 3 times with similar results.

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TABLE 1. **Validation of select gene targets regulated by FBLN5 expression in 3T3-L1 fibroblasts.** Among the genes whose expression was controlled by FBLN5 as detected in microarray analyses, the expression of 13 genes was confirmed by semi-quantitative real-time PCR.

Gene Name	NCBI number	Microarray Expression	Real-time PCR Expression
Unknown	BB503935	9.2-fold decrease	22.2-fold decrease
Unknown	BB533736	5.4-fold increase	3.2-fold increase
Unknown	BB831146	9.2-fold increase	2.1-fold increase
Pleckstrin homology domain-containing family A member	BC010215	7.5-fold decrease	5.8-fold decrease
Transglutaminase 2	BC016492	5.8-fold decrease	33.6-fold decrease
Homeobox D9	BC019150	9.4-fold increase	2.1-fold increase
Rho GTPase-activating protein 24	BC025502	5.5-fold decrease	3.3-fold decrease
Thrombospondin 1	M87276	10.7-fold increase	10.5-fold increase
Procollagen, type XI, α 1	NM_007729	9.9-fold increase	2.3-fold increase
Angiopoietin 1	NM_009640	8.3-fold increase	2.1-fold increase
Cysteine-rich protein 61	NM_010516	10.4-fold increase	1.8-fold increase
Dickkopf homolog 3	NM_015814	6.9-fold increase	6.7-fold increase
Fibromodulin	NM_021355	9.6-fold increase	10.6-fold increase

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Figure 1.

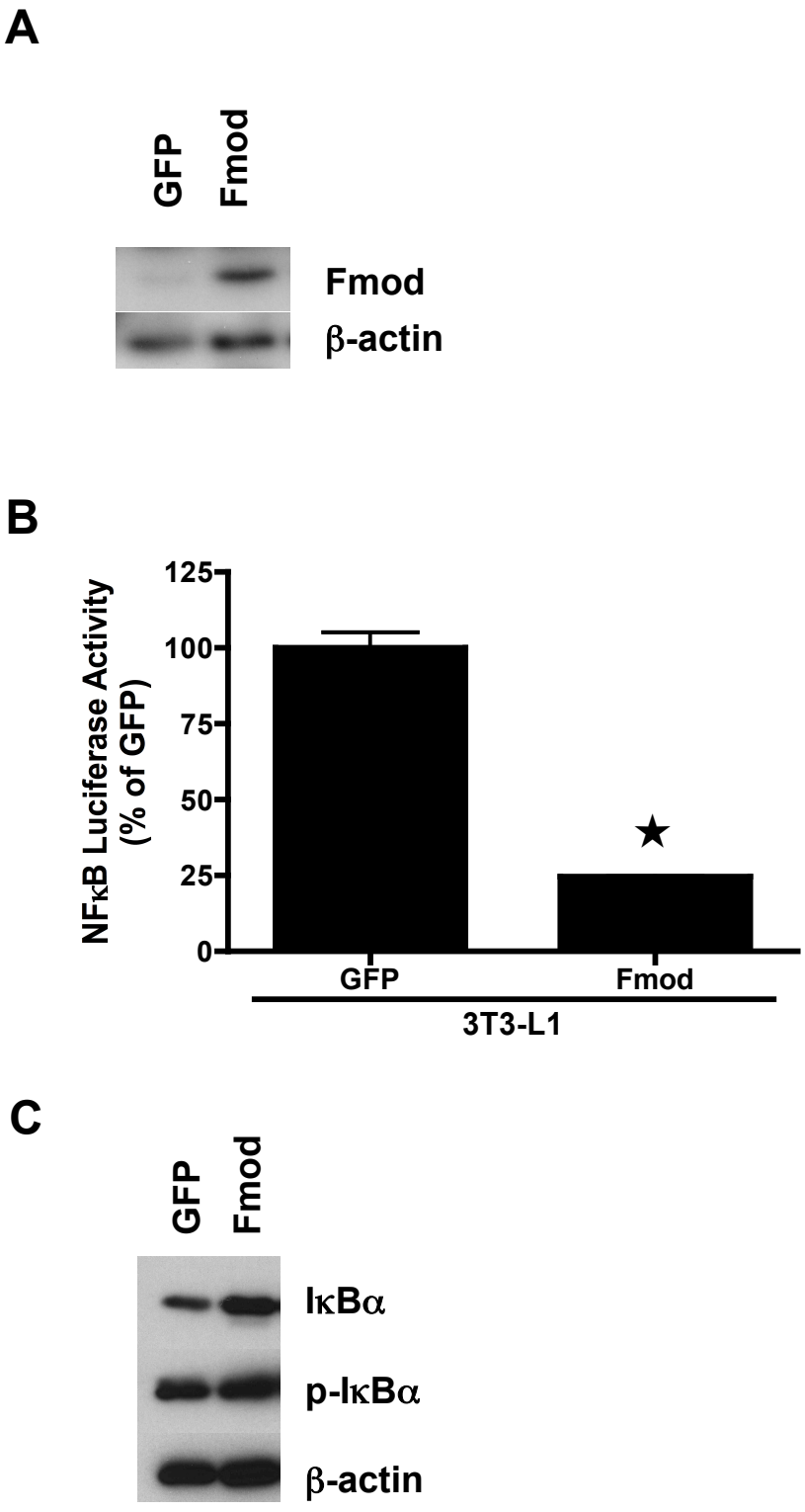

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Figure 2.

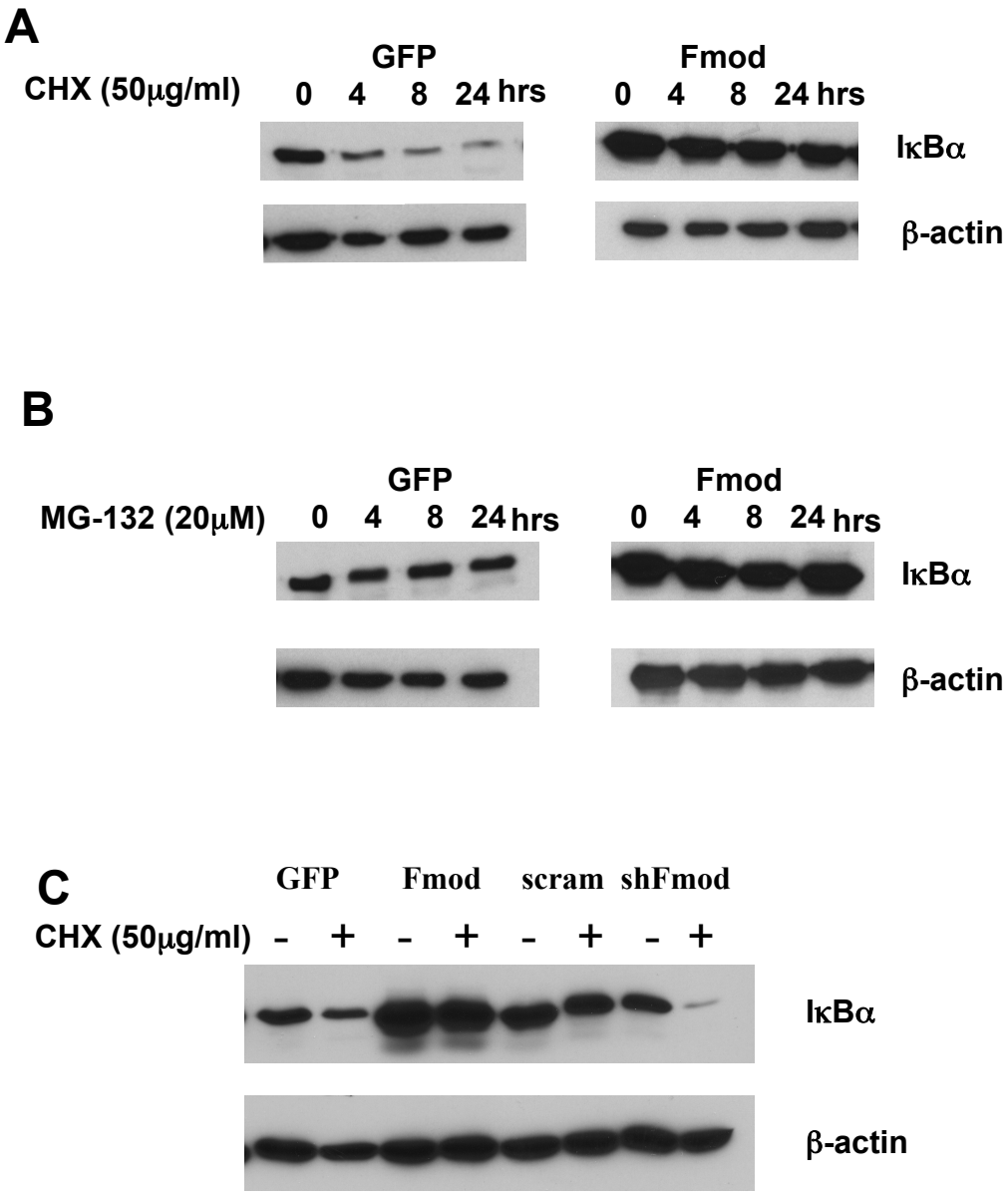

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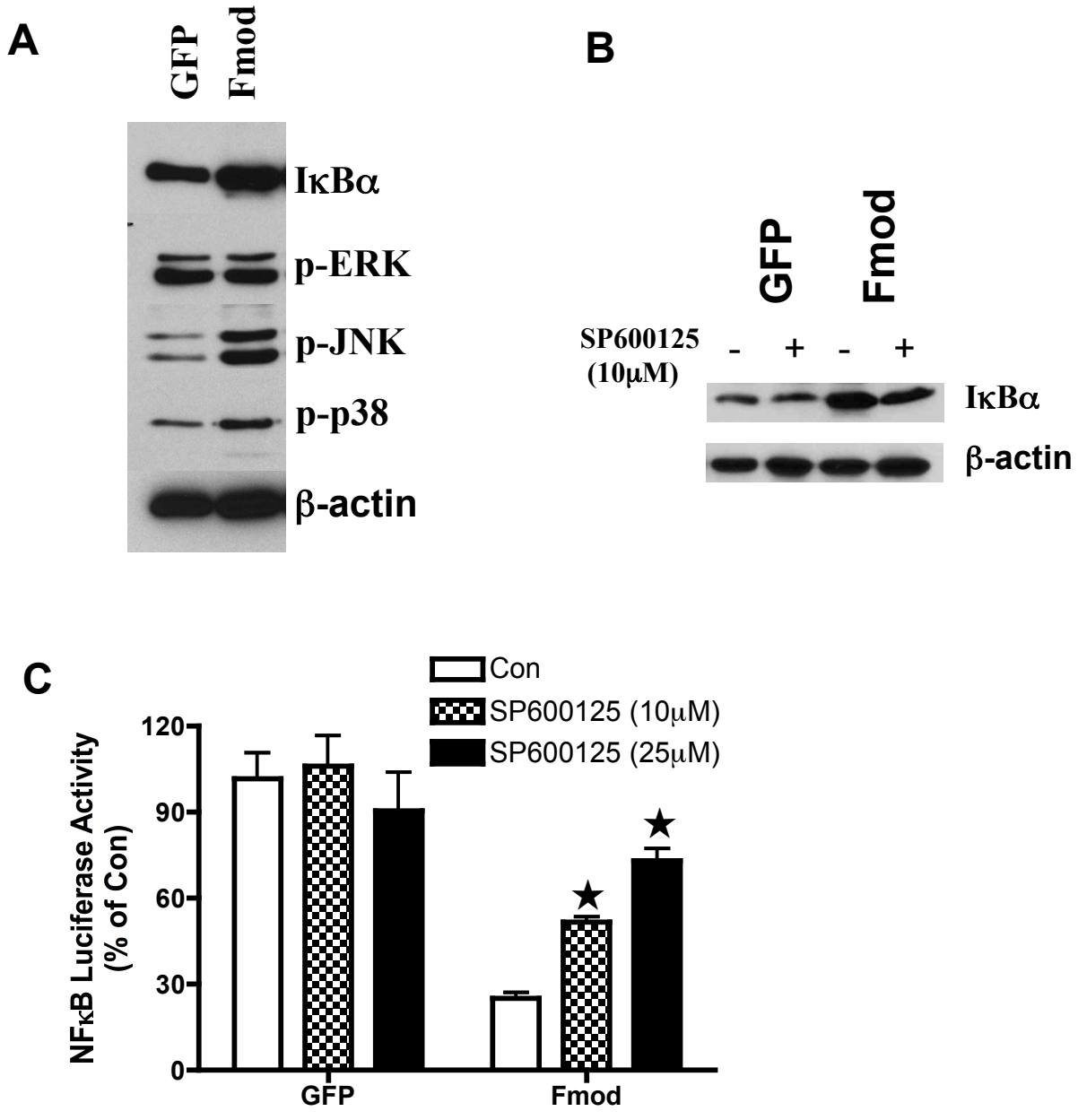
Figure 3.



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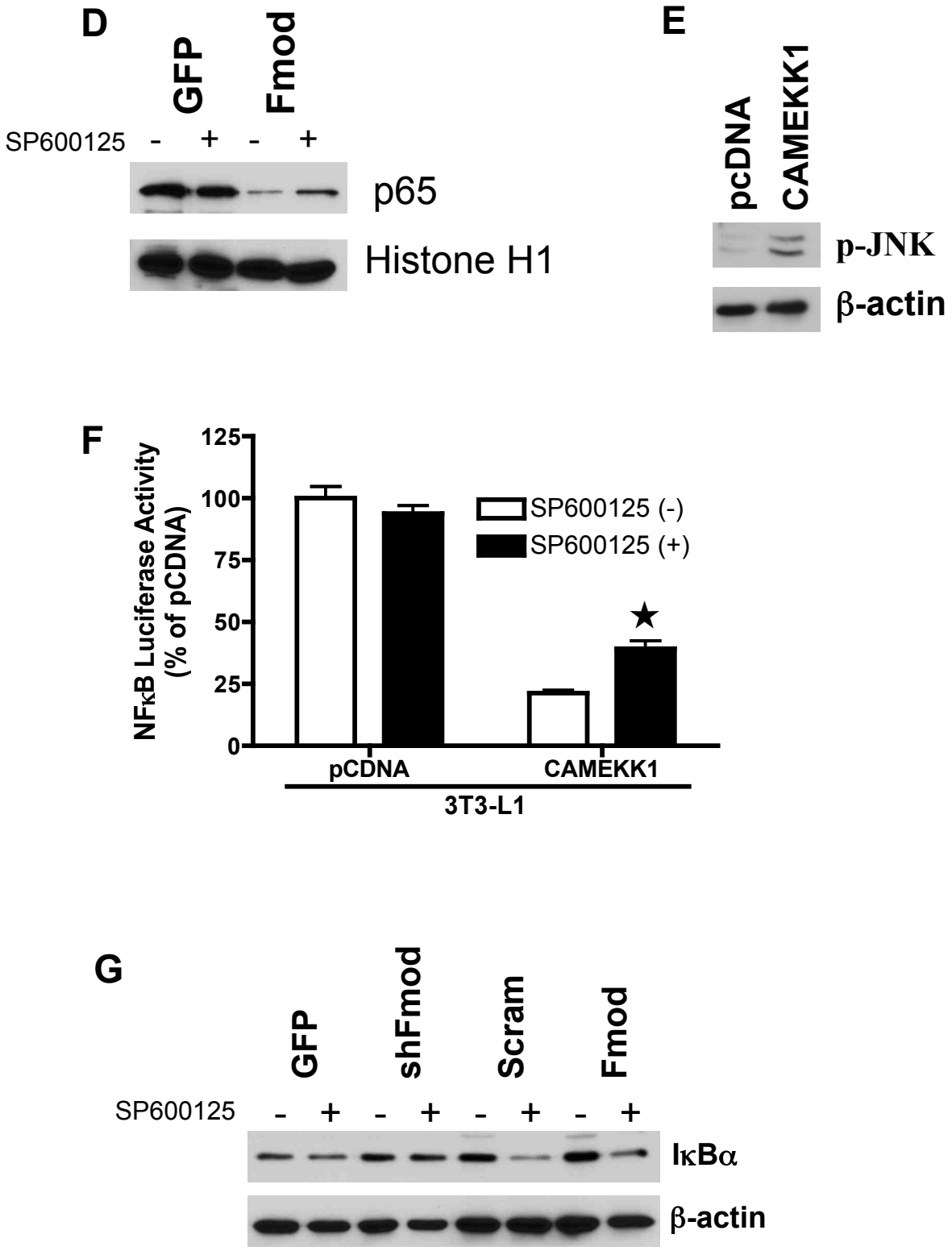

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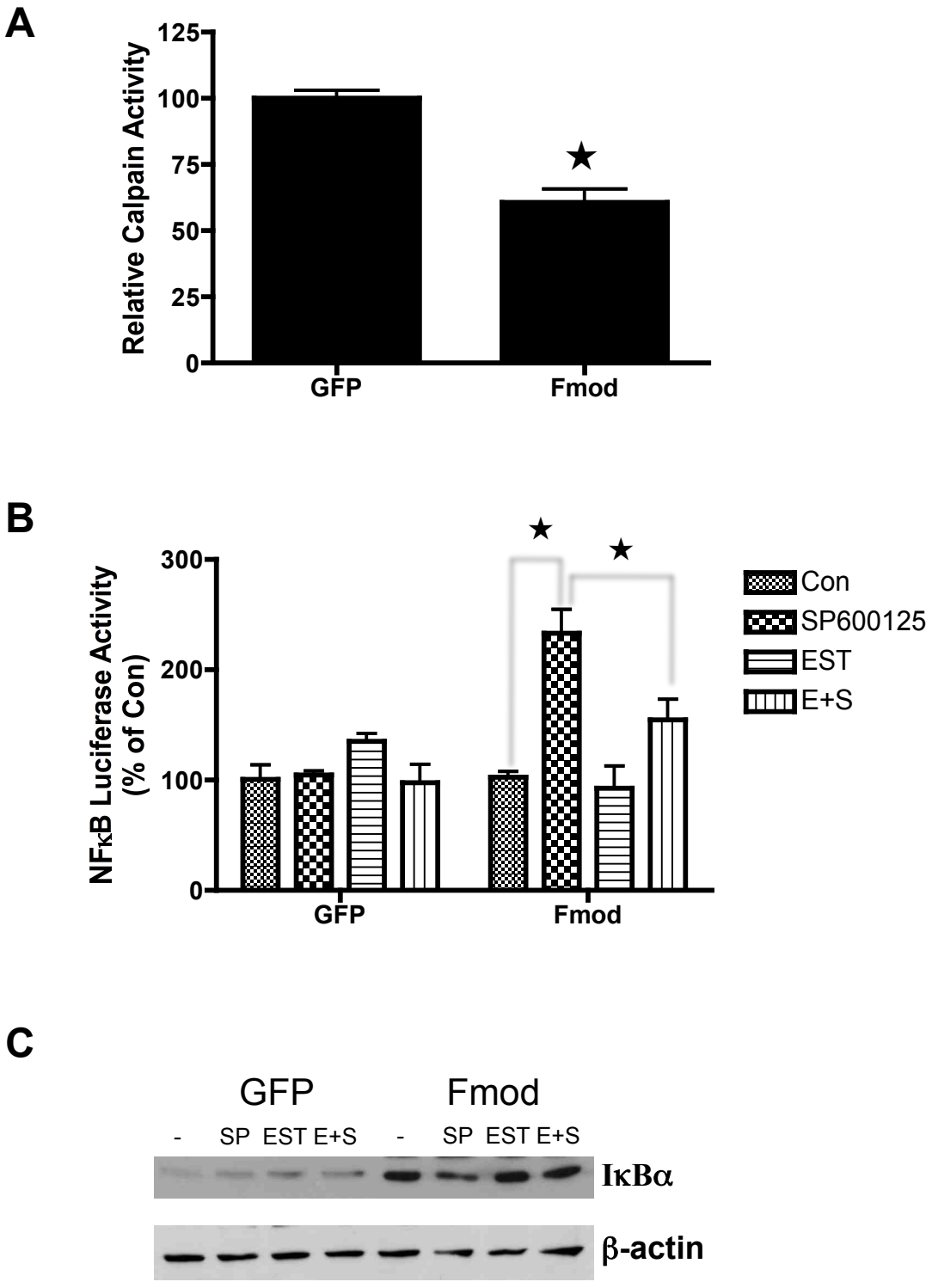

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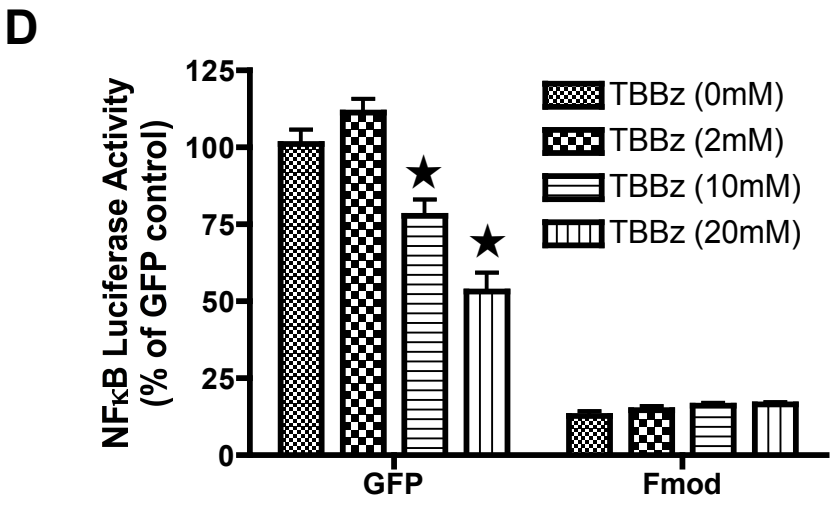

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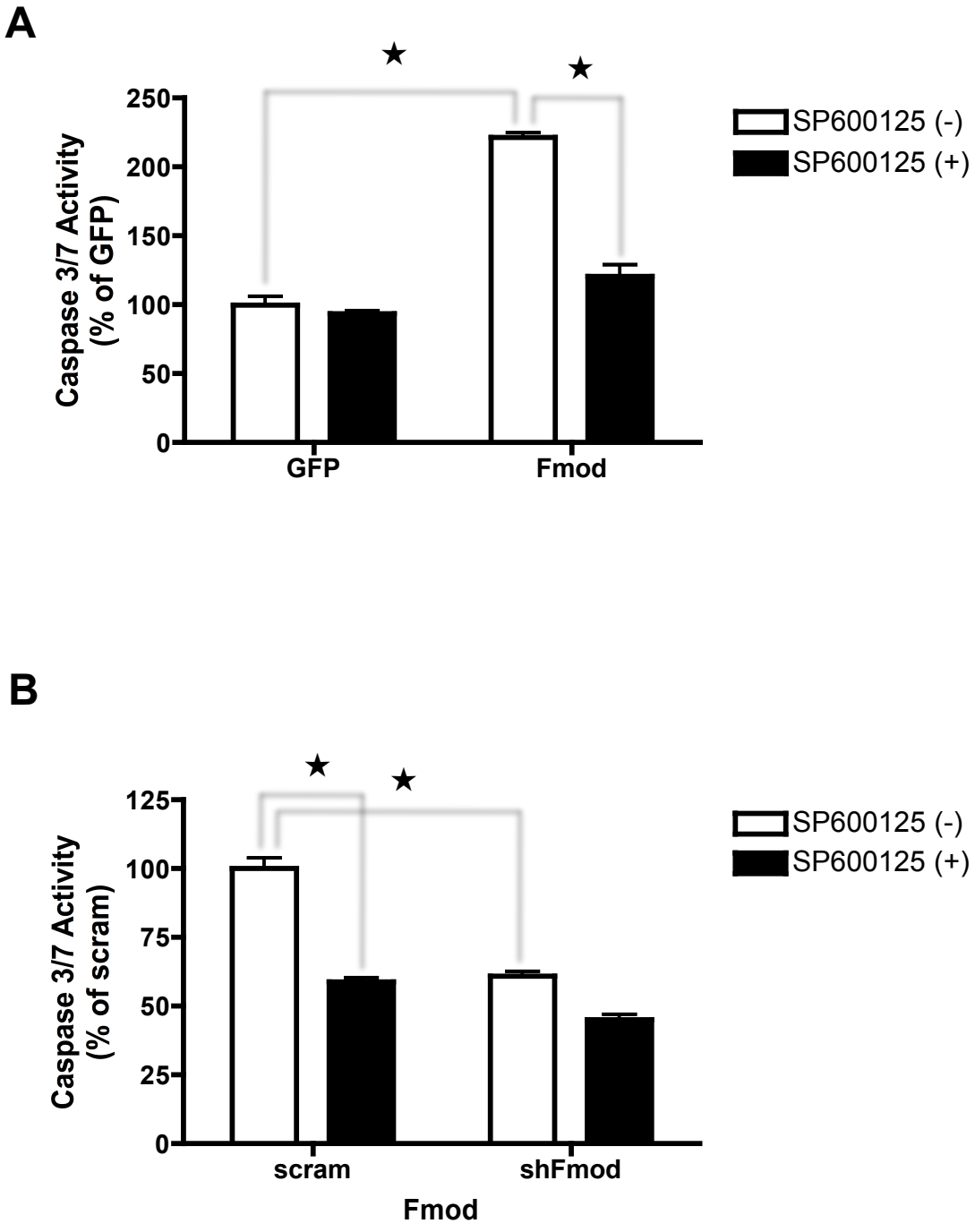

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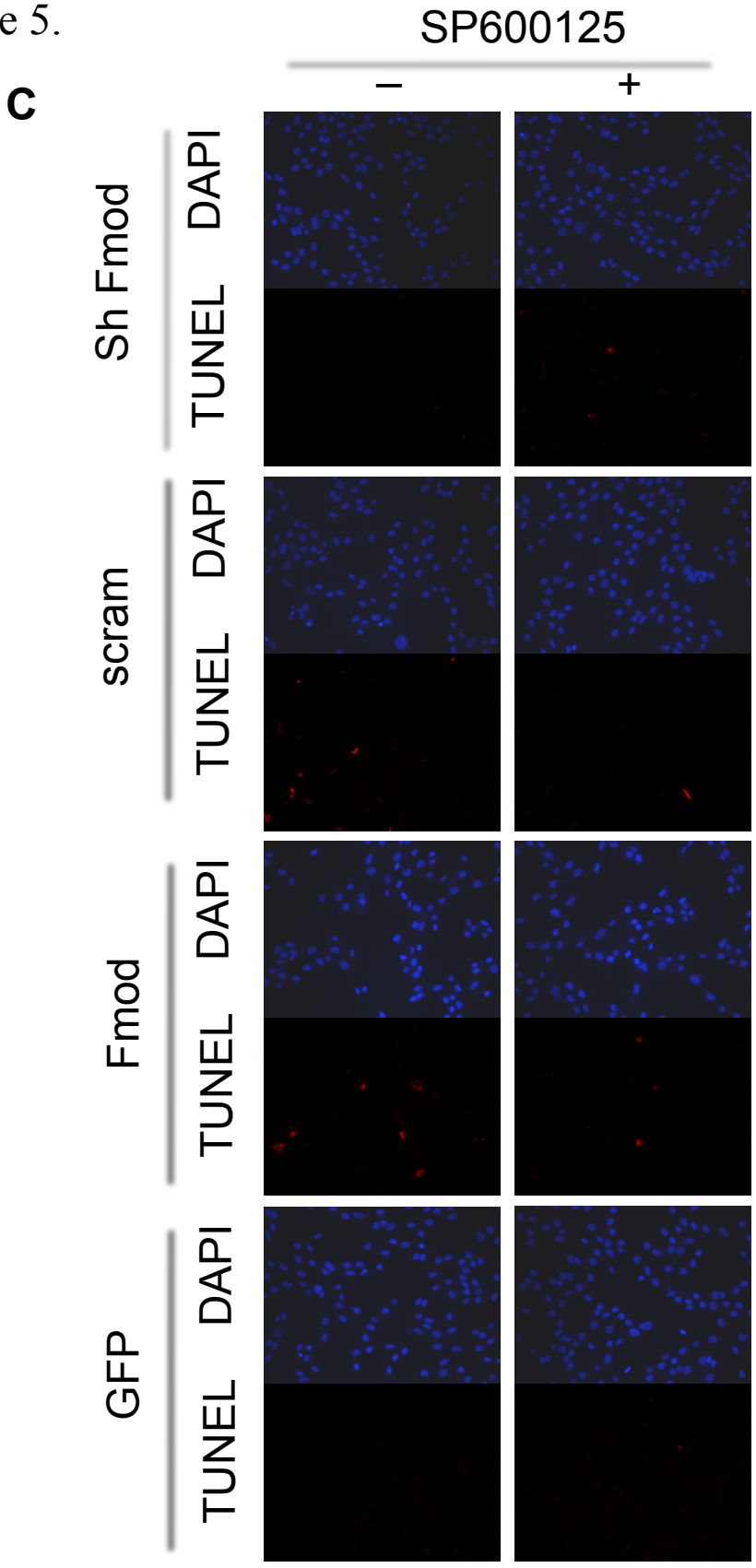
Figure 5.



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